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(54) Title: USE OF BACTERICIDAL/PERMEABILITY INCREASING PROTEIN OR BIOLOGICALLY ACTIVE ANALOGS THEREOF TO TREAT LIPOPOLYSACCHARIDE ASSOCIATED GRAM NEGATIVE INFECTIONS

(57) Abstract

The present invention provides a method of inhibiting lipopolysaccharide (LPS)-mediated stimulation of cells. This method comprises contacting the cells, in the presence of a cell-stimulating amount of lipopolysaccharide, with Bactericidal/Permeability Increasing Protein (BPI) in an amount effective to inhibit cell stimulation.

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USE OF BACTERICIDAL/PERMEABILITY INCREASING PROTEIN OR BIOLOGICALLY ACTIVE ANALOGS THEREOF TO TREAT LIPOPOLYSACCHARIDE ASSOCIATED GRAM NEGATIVE INFECTIONS

Background of the Invention

Gram negative infections are a major cause of morbidity and mortality especially in hospitalized and immunocompromised patients. [Duma, R.J., Am. J. of Med., 78 (Suppl. 6A): 154-164 (1985); and Kreger B.E., D.E. Craven and W.R. McCabe, Am. J. Med., 68: 344-355 (1980)]

Although available antibiotics are effective in containing the infection, they do nothing to neutralize the pathophysiological effects associated with lipopolysaccharide (LPS). LPS, or endotoxin, is a major component of the outer membrane of gram negative bacteria and is released when the organisms are lysed. [Ahenep, J.L. and K.A. Morgan, J. Infect. Dis., 150 (3): 380-388 (1984)]

LPS released during antibiotic therapy is a potent stimulator of the inflammatory response. Many detrimental effects of LPS in vivo result from soluble mediators released by inflammatory cells. [Morrison D.C. and R.J. Ulevich, Am. J. Pathol., 93 (2): 527-617 (1978)] LPS induces the release of mediators by host inflammatory cells which may ultimately result in disseminated intravascular coagulation (DIC), adult respiratory distress syndrome (ARDS), renal failure, and irreversible shock.

Monocytes and neutrophilic granulocytes play a key role in host defense against bacterial infections and also participate in the pathology of endotoxemia. These cells ingest and kill microorganisms intracellularly and also respond to LPS in vivo and in vitro by releasing soluble proteins with microbicidal, proteolytic, opsonic, pyrogenic, complement activating and tissue damaging effects. Tumor necrosis factor (TNF), a cytokine released by LPS stimulated monocytes mimics some of the toxic effects of LPS in vivo. Injecting animals with TNF causes fever, shock and alterations in glucose metabolism. TNF is also a potent stimulator of neutrophils.

Soluble LPS causes decreased neutrophil chemotaxis, increased adhesiveness, elevated hexose monophosphate shunt activity and O_2 radical production, upregulation of surface receptors for complement, and release of granule proteins into the surrounding medium. [Morrison and Ulevich (1978)]

Both specific and azurophil compartments degranulate in response to LPS. [Bannatyne, R.M., N.M. Harnett, K.Y. Lee and W.D. Rigger, J. Infect. Dis., 156 (4): 469-474 (1977)] Azurophil proteins released in response to LPS may be both harmful and beneficial to the host. Neutrophil elastase causes degradation of protease inhibitors responsible for suppressing the coagulation cascade. This results in coagulopathies such as disseminated intravascular coagulation, a potentially lethal consequence of endotoxemia. Azurophil granules also contain bactericidal molecules such as myeloperoxidase and Bactericidal/Permeability Increasing Factor (BPI).

Rabbit BPI was first discovered in 1975. [Weiss, J., R.C. Franson, S. Becherdite, K. Schmeidler, and P. Elsbach, J. Clin. Invest., 55:33 (1975)] BPI was isolated from human neutrophils in 1978. [Weiss, J., P. Elsbach, I. Olson and H. Odeberg, J. Biol. Chem, 253 (8): 2664-2672 (1978)].

In 1984 a 57 kD protein with similar properties was isolated from human neutrophils. [Shafer, W.M., C.E. Martin and J.K. Spitznagel, Infect. Immun., 45:29 (1984)] This protein is identical to BPI by N-Terminal sequence amino acid composition, molecular weight and source. Although, the authors were unable to reproduce the chromatographic isolation procedure used by Elsbach, et al. and Weiss, et al.

Human BPI is a 57 kD protein which binds to the outer membrane of susceptible gram negative bacteria. [Weiss, et al. (1978)] The fact that BPI is a Lipid A binding protein is evidenced by: (1) rough strains of bacteria are more sensitive to both bactericidal and permeability increasing activities of BPI [Weiss, J., M. Hutzler and L. Kao, Infect. Immun., 51:594 (1986)]; (2) mutations in Lipid A cause decreased binding and increase resistance to bactericidal activity of both polymyxin B and BPI [Farley, M.M., W.M. Shafer and J.K. Spitznagel, Infect. Immun., 56:1589 (1988)]; (3) BPI competes with polymyxin B for binding to S. typhimurium [Farley 1988]; (4) BPI has sequence homology and immunocrossreactivity to another LPS binding protein Lipopolysaccharide Binding Protein (LBP). LBP-LPS complexes have been shown to stimulate the oxidative burst on neutrophils in response to formylated peptides. High density lipoprotein (HDL), another LPS binding protein, found in human serum in complex with LPS does not show the stimulatory effect on neutrophils. BPI binding disrupts LPS structure, alters microbial permeability to small hydrophobic molecules and causes cell death (Weiss, et al., 1978). BPI kills bacteria under physiologic conditions of pH and ionic strength in vitro indicating that it may be active in vivo outside the low pH environment of the phagolysosome. All of the bactericidal and permeability increasing activities of BPI are present in the N-terminal 25kD fragment of the protein. [Ooi, C.E., J. Weiss, P. Elsbach, B. Frangione, and B. Marrion, J. Biol. Chem., 262: 14891 (1987)] Prior to the subject invention,

however, it has been understood that the beneficial effects of BPI are limited to its bactericidal effects.

Despite improvements in antibiotic therapy, morbidity and mortality associated with endotoxemia remains high. Antibiotics alone are not effective in neutralizing the toxic effects of LPS. Therefore, the need arises for an adjunct therapy with direct LPS neutralizing activity. Current methods for treatment of endotoxemia use antibiotics and supportive care. Most available adjunct therapies treat symptoms of endotoxic shock such as low blood pressure and fever but do not inactivate endotoxin. Other therapies inhibit inflammatory host responses to LPS. As indicated below, present therapies have major limitations due to toxicity, immunogenicity, or irreproducible efficacy between animal models and human trials.

Polymyxin B is a basic polypeptide antibiotic which has been shown to bind to, and structurally disrupt, the most toxic and biologically active component of endotoxin, Lipid A. Polymyxin B has been shown to inhibit LPS activation of neutrophil granule release in vitro and is an effective treatment for gram negative sepsis in humans. However, because of its systemic toxicity, this drug has limited use except as a topical agent.

Combination therapy using antibiotics and high doses of methylprednisolone sodium succinate (MPSS) has been shown to prevent death in an experimental model of gram negative sepsis using dogs. Another study using MPSS with antibiotics in a multicenter, double blind, placebo-controlled, clinical study in 223 patients with clinical signs of systemic sepsis concluded that mortality was not significantly different between the treatment and placebo groups. Further, the investigators found that resolution of secondary infection within 14 days was significantly higher in the placebo group.

A relatively new approach to treatment of endotoxemia is passive immunization with endotoxin neutralizing antibodies. Hyperimmune human immunoglobulin against E. Coli J5 has been shown to reduce mortality in patients with gram negative bacteremia and shock by 50%. Other groups have shown promising results in animal models using mouse, chimeric, and human monoclonal antibodies. Although monoclonal antibodies have advantages over hyperimmune sera, e.g. more consistent drug potency and decreased transmission of human pathogens, there are still many problems associated with administering immunoglobulin to neutralize LPS. Host responses to the immunoglobulins themselves can result in hypersensitivity. Tissue damage following complement activation and deposition of immune complexes is another concern in the use of therapies involving anti-endotoxin antibodies in septic patients. Also, immunoglobulins are large molecules, especially the pentameric IgMs currently in clinical trials, and are rapidly cleared by the reticuloendothelial system, diminishing the half-life of the drug.

Endotoxins elicit responses which are beneficial as well as damaging to the host. Endotoxemia induces production of LPS binding proteins from the liver and causes release of microbicidal proteins from leukocytes. In applicants' studies of neutrophil proteins involved in host defense, it has been determined that one of these proteins, BPI, is not only a potent microbicidal agent in vitro, but it also interferes with the ability of LPS to stimulate neutrophils. Specifically, it has been demonstrated that BPI binds to soluble LPS and neutralizes its ability to activate neutrophils. Accordingly, this invention provides a therapeutic method for the treatment of LPS toxicity in gram negative septicemia.

Summary of the Invention

The present invention provides a method of inhibiting lipopolysaccharide (LPS)-mediated stimulation of cells. This method comprises contacting the cells, in the presence of a cell-stimulating amount of lipopolysaccharide, with Bactericidal/Permeability Increasing Protein (BPI) in an amount effective to inhibit cell stimulation.

The invention further provides a method of treating a gram negative bacterial infection. This method comprises contacting the bacterial infection with purified BPI or a biologically active polypeptide analog thereof in an amount effective to inhibit LPS-mediated stimulation of cells and thereby treat the bacterial infection.

Additionally, the present invention provides for a composition for treatment of a gram negative bacterial infection. This composition comprises purified BPI or a biologically active polypeptide analog thereof in an amount effective to inhibit LPS-mediated stimulation of cells and a suitable carrier.

The present invention additionally provides a method of treating a subject suffering from endotoxin-related shock caused by a gram negative bacterial infection which comprises administering to the subject an amount of BPI effective to combat the gram negative infection and treat the subject so as to alleviate the endotoxin-related shock.

Further, the invention provides a method of treating a subject suffering from disorder involving disseminated intravascular coagulation. The method comprises administering to the subject an amount of BPI effective to alleviate the symptoms of disseminated intravascular coagulation and thereby treat the subject.

Further, the present invention provides a method of treating a subject suffering from endotoxemia caused by a gram negative infection which comprises administering to the subject an amount of BPI effective to combat the gram negative bacterial infection and treat the subject suffering from endotoxemia.

As used herein endotoxemia means a condition in which the blood contains poisonous products, either those produced by the body cells or those resulting from microorganisms, i.e. gram negative bacteria.

The invention also provides a method of treating a subject suffering from endotoxin-related anemia caused by a gram negative bacterial infection which comprises administering to the subject an amount of BPI effective to combat the gram negative bacterial infection and treat the subject so as to alleviate endotoxin-related anemia.

The present invention further provides a method of treating a subject suffering from endotoxin-related leukopenia caused by a gram negative bacterial infection. The method comprises administering to the subject an amount of BPI effective to combat the gram negative bacterial infection and treat the subject so as to alleviate endotoxin-related leukopenia. Further, the invention includes a method of treating a subject suffering from endotoxin-related thrombocytopenia caused by a gram negative bacterial infection which comprises administering to the subject an amount of BPI effective to combat the gram negative bacterial infection and treat the subject so as to alleviate endotoxin-related thrombocytopenia.

The invention also provides a method of inhibiting a pyrogen which comprises contacting the pyrogen with an amount of BPI so as to inhibit the pyrogen.

Furtherm re, the invention also includes a method of inhibiting lipopolysaccharide-mediated tumor necrosis factor production by cells which comprises contacting the cells in the presence of a cell-stimulating amount of lipopolysaccharide, with BPI in an amount effective to inhibit lipopolysaccharide-mediated tumor necrosis factor production by cells.

The invention also includes a method of inhibiting gram negative bacteria-mediated tumor necrosis factor production by cells which comprises contacting the gram negative bacteria with BPI in an amount effective to inhibit gram negative-mediated tumor necrosis factor production by cells.

Moreover, the invention includes a composition for the treatment of a subject suffering from endotoxin-related shock. The composition comprises a purified BPI or a biologically active polypeptide analog thereof in an amount effective to treat a subject suffering from endotoxin-related shock and a suitable carrier.

Further, the invention includes a composition for the treatment of a subject suffering from disseminated intravascular coagulation. The composition comprises a purified BPI or a biologically active polypeptide analog thereof in an amount effective to treat a subject suffering from disseminated intravascular coagulation and a suitable carrier.

The invention also includes a composition for the treatment of a subject suffering from endotoxemia comprising a purified BPI or a biologically active polypeptide analog thereof in an amount effective to treat a subject suffering from endotoxemia and a suitable carrier.

The invention additionally provides a composition for the treatment of a subject suffering from endotoxin-related anemia comprising purified BPI or a biologically

active polypeptide analog thereof in an amount effective to treat a subject suffering from endotoxin-related anemia and a suitable carrier.

Additionally, the invention provides a composition for the treatment of a subject suffering from endotoxin-related leukopenia. The composition comprises purified BPI or a biologically active polypeptide analog thereof in an amount effective to treat a subject suffering from endotoxin-related leukopenia and a suitable carrier. Further provided is a composition for the treatment of a subject suffering from endotoxin-related thrombocytopenia. The composition comprises purified BPI or a biologically active polypeptide analog thereof in an amount effective to treat a subject suffering from endotoxin-related thrombocytopenia and a suitable carrier.

As used herein endotoxin-related leukopenia is a condition associated with a gram negative bacterial infection, the manifestation of which is a decrease below the normal number of leukocytes in the peripheral blood. Moreover, as used herein endotoxin-related thrombocytopenia is a condition associated with a gram negative bacterial infection, the manifestation of which is a decrease below the normal number of thrombocytes.

Also, the invention provides a composition for inhibiting lipopolysaccharide-mediated tumor necrosis factor production by cells comprising purified BPI or a biologically active polypeptide analog thereof in an amount effective to inhibit lipopolysaccharide-mediated tumor necrosis factor production by cells and a suitable carrier. Moreover, the invention provides a composition for inhibiting gram negative bacteria-mediated tumor necrosis factor production by cells comprising purified BPI or a biologically active polypeptide analog thereof in an amount effective to inhibit gram negative bacteria-mediated tumor necrosis factor production by cells and a suitable carrier.

The invention provides a composition for inhibiting a pyrogen. The composition comprises purified BPI or a biologically active polypeptide analog thereof in an amount effective to inhibit a pyrogen.

Additionally, the invention provides a method of preventing a symptom associated with a gram negative bacterial infection in a subject which comprises administering to the subject an amount of Bactericidal/Permeability Increasing Protein effective to prevent the gram negative bacterial infection and thereby prevent the symptom.

Moreover, also provided is a method of preventing a disorder involving disseminated intravascular coagulation in a subject which comprises administering to the subject an amount of Bactericidal/Permeability Increasing Protein effective to prevent the symptoms of disseminated intravascular coagulation and thereby preventing the disorder.

Finally, the invention provides a method of isolating and recovering purified Bactericidal/Permeability Increasing Protein which comprises: (a) obtaining a crude sample of Bactericidal/Permeability Increasing Protein; and (b) separating the crude sample by column chromatography using de-pyrogenated solutions thereby isolating and recovering purified Bactericidal/Permeability Increasing Protein.

Brief Description of the Figures

Figure 1a: Mean fluorescence intensity of CR1 on freshly isolated neutrophils was measured by FACS analysis. Cells were stimulated with varying doses of E. Coli 0111:B4 LPS as described in Materials and Methods. Since mean fluorescence intensity varies between individuals, the data is expressed as percent of the maximum response observed. Data shown represents the mean +/- Standard Error of three experiments.

Figure 1b:0111:B4 LPS (10 ng/ml) was preincubated with varying doses of crude azurophil extract for 30 minutes at 37°C prior to testing for neutrophil stimulation. Data shown represents the mean +/- Standard Error of duplicates from a representative experiment. Values are expressed as % inhibition of the response to LPS alone.

Figure 2: Crude azurophil extract was separated by reverse phase HPLC. Each peak was collected manually and protein concentrations were determined by amino acid analysis. An aliquot (1μg) of each peak was dried in the presence of low endotoxin BSA, then redried in the presence of pyrogen free 0.1% acetic acid. Data shown represent the mean +/- Standard Error of duplicates from a representative experiment.

Figure 3a:BPI purified by size exclusion followed by cation exchange HPLC was subjected to reverse phase HPLC and fractions were tested for LPS inhibitory activity.

Figure 3b: Data show the RPLC profile of the 2X purified material along with the inhibitory activity and SDS PAGE analysis of fractions 20,21 and 22.

Figure 4:0111:B4 LPS (10 ng/ml) was preincubated with varying doses of (A) purified BPI, and (B) polymyxin B, then tested for neutrophil stimulatory activity. Results from two experiments shows inhibition of complement receptor expression on neutrophils with Standard Errors for replicate samples.

Figure 5: (a) A bar graph illustrating BPI expression on the surface of neutrophils stimulated with FMLP, TNF, and LPS. (b) A bar graph illustrating maximal CR3 upregulation of human neutrophil cell surface expression.

Figure 6: A bar graph illustrating that BPI and polymyxin B inhibited more than 70% at time=0 of the neutrophil response to LPS.

Figure 7: A graph illustrating that BPI inhibits LPS activity on LAL assay.

Figure 8: A chromatogram showing a fractionated azurophile granule extract by cation exchange HPLC (step 1); the dotted line traces LPS inhibitory activity and the solid line traces protein absorbance.

Figure 9: A chromatogram showing a fractionated azurophile granule extract by cation exchange HPLC (step); the dotted line traces LPS inhibitory activity and the solid line traces protein absorbance.

Figure 10: A chromatogram showing a fractionated azurophile granule extract by size exclusion HPLC (step 3); the dotted line traces LPS inhibitory activity and the solid line traces protein absorbance.

Figure 11: An SDS-PAGE gel of the azurophil granule extract, the precipitated extract, and fraction pools from the three chromatographic steps.

Figure 12: Analysis of purified BPI by microbore reverse phase HPLC identifying a single major peak which accounts for 97% of the total protein.

Figure 13: A line graph illustrating inhibition of the neutrophil response to 10 ng/ml LPS by BPI.

Figure 14: A line graph showing BPI directly binds to LPS.

Figure 15: A line graph showing BPI binding to immobilized LPS was inhibited by polymyxin B.

Figure 16: A line graph showing that BPI binds to LPS in the presence of plasma.

Figure 17: A line graph showing BPI binds to LPS in the presence of serum.

Figure 18: A bar graph showing that BPI modulates pyrogenic response to LPS.

<u>Detailed Description of the Invention</u>

The present invention provides a method of inhibiting lipopolysaccharide (LPS)-mediated stimulation of cells. This method comprises contacting the cells, in the presence of a cell-stimulating amount of lipopolysaccharide, with BPI in an amount effective to inhibit cell stimulation.

The amount of BPI effective to inhibit cell stimulation will vary according to the conditions present. The amount effective to inhibit cell stimulation is preferably from about 100 ng to about 100 mg, with the most preferred amount being from about 10 μg to about 10 mg.

Neutrophils and monocytes are the cells of greatest importance with regard to the application of the subject invention. However, other cells such as endothelial cells are also affected by LPS and may be used in this invention.

In the preferred embodiment purified BPI is used. BPI also comprises recombinant BPI and biologically active polypeptide analogs thereof. One suitable analog of BPI comprises a polypeptide which has a molecular weight of about 25kD and corresponds to the N-terminal amino acid sequence of BPI.

As used herein a biologically active polypeptide analog of Bactericidal/Permeability Increasing Protein means a polypeptide which has substantially the same amino acid sequence as, and the biological activity of, native or naturally-occurring Bactericidal/Permeability Increasing Protein.

The invention further provides a method of treating a gram negative bacterial infection. This method comprises contacting the bacterial infection with purified

BPI or a biologically active polypeptide analog thereof in an amount effective to inhibit LPS-mediated stimulation of cells and thereby treat the bacterial infection.

It would be clear to one skilled in the art that gram negative bacterial infection includes gram negative sepsis, the most common nosocomial infection which causes death. Generally, gram negative sepsis is a severe toxic, febrile state resulting from infection with pyogenic microorganisms, with or without septicemia.

The gram negative bacterial infection may be associated with endotoxic shock or an inflammatory condition. The inflammatory condition may, for example, be associated with disseminated intravascular coagulation (DIC), adult respiratory distress syndrome (ARDS), or renal failure.

In the preferred embodiment the gram negative bacterial infection is present in a subject, most preferably, a human being.

Additionally, the present invention provides for a composition for treatment of a gram negative bacterial infection. This composition comprises purified BPI or a biologically active polypeptide analog thereof in an amount effective to inhibit LPS-mediated stimulation of cells and a suitable carrier.

In the preferred embodiment the BPI or a biologically active polypeptide analog thereof is administered in a pharmaceutically acceptable carrier. Pharmaceutically acceptable carrier encompasses any of the standard pharmaceutical carriers such as sterile solution, tablets, coated tablets and capsules. Typically such carriers contain excipients such as starch, milk, sugar, certain types of clay, gelatin, stensic acid, talc, vegetable fats or oils, gums, glycols, or other known excipients. Such carriers may also include flavor and color additives or other ingredients. Compositions comprising such carriers are formulated by well known conventional

methods. However, the composition comprising BPI or a biologically active polypeptide analog thereof in an amount effective t suppress LPS mediated stimulation of neutrophils or monocytes is previously unknown.

In this method, the administration of the composition may be effected by any of the well known methods, including but not limited to, oral, intravenous, intramuscular, and subcutaneous administration.

In the practice of the method of this invention the amount of BPI or a biologically active polypeptide analog thereof incorporated in the composition may vary widely. Methods for determining the precise amount are well known to those skilled in the art and depend inter alia upon the subject being treated, the specific pharmaceutical carrier and route of administration being employed, and the frequency with which the composition is to be administered.

The present invention additionally provides a method of treating a subject suffering from endotoxin-related shock caused by a gram negative bacterial infection which comprises administering to the subject an amount of BPI effective to combat the gram negative infection and treat the subject so as to alleviate the endotoxin-related shock. Endotoxins, as used herein, are substances containing lipopolysaccharide complexes found in the cell walls of microorganisms, principally gram-negative bacteria.

Further, the invention provides a method of treating a subject suffering from disorder involving disseminated intravascular coagulation. The method comprises administering to the subject an amount of BPI effective to alleviate the symptoms of disseminated intravascular coagulation and thereby treat the subject.
As used herein, the term disseminated intravascular coagulation is a complex disorder of the clotting mechanisms, in which coagulation factors are consumed

at an accelerated rate, with generalized fibrin deposition and thrombosis, hemorrhages, and further depletion of the coagulation factors. Moreover, disseminated intravascular coagulation may be acute or chronic.

Further, the present invention provides a method of treating a subject suffering from endotoxemia caused by a gram negative infection which comprises administering to the subject an amount of BPI effective to combat the gram negative bacterial infection and treat the subject suffering from endotoxemia.

As used herein endotoxemia means a condition in which the blood contains poisonous products, either those produced by the body cells or those resulting from microorganisms, i.e. gram negative bacteria.

The invention also provides a method of treating a subject suffering from endotoxin-related anemia caused by a gram negative bacterial infection which comprises administering to the subject an amount of BPI effective to combat the gram negative bacterial infection and treat the subject so as to alleviate endotoxin-related anemia.

The present invention further provides a method of treating a subject suffering from endotoxin-related leukopenia caused by a gram negative bacterial infection. The method comprises administering to the subject an amount of BPI effective to combat the gram negative bacterial infection and treat the subject so as to alleviate endotoxin-related leukopenia. Further, the invention includes a method of treating a subject suffering from endotoxin-related thrombocytopenia caused by a gram negative bacterial infection which comprises administering to the subject an amount of BPI effective to combat the gram negative bacterial infection and treat the subject so as to alleviate endotoxin-related thrombocytopenia.

The invention also provides a method of inhibiting a pyrogen which comprises contacting the pyrogen with an amount of BPI so as to inhibit the pyrogen. As used herein, a pyrogen is any fever-producing substance; exogenous pyrogens include bacterial endotoxins, especially of gram-negative bacteria; endogenous pyrogen is a thermolabile protein derived from such cells as mononuclear leukocytes which acts on the brain centers to produce fever.

Furthermore, the invention also includes a method of inhibiting lipopolysaccharide-mediated tumor necrosis factor production by cells which comprises contacting the cells in the presence of a cell-stimulating amount of lipopolysaccharide, with BPI in an amount effective to inhibit lipopolysaccharide-mediated tumor necrosis factor production by cells.

The invention also includes a method of inhibiting gram negative bacteria-mediated tumor necrosis factor production by cells which comprises contacting the gram negative bacteria with BPI in an amount effective to inhibit gram negative-mediated tumor necrosis factor production by cells.

The amount of BPI effective to inhibit cell stimulation will vary according to the conditions present. The amount effective to inhibit cell stimulation is preferably from about 100 ng to about 100 mg, with the most preferred amount being from about 10μg to about 10 mg.

In the above-described methods, the BPI comprises recombinant BPI or a biologically active polypeptide analog thereof. Moreover, the biologically active polypeptide analog of BPI comprises a polypeptide which has a molecular weight of about 25 kD and corresponds to the N-terminal amino acid sequence of BPI.

Moreover, the invention includes a composition for the treatment of a subject

suffering from ndotoxin-related shock. The composition comprises a purified BPI or a biologically active polypeptide analog th reof in an amount effective to treat a subject suffering from endotoxin-related shock and a suitable carrier.

Further, the invention includes a composition for the treatment of a subject suffering from disseminated intravascular coagulation. The composition comprises a purified BPI or a biologically active polypeptide analog thereof in an amount effective to treat a subject suffering from disseminated intravascular coagulation and a suitable carrier.

The invention also includes a composition for the treatment of a subject suffering from endotoxemia comprising a purified BPI or a biologically active polypeptide analog thereof in an amount effective to treat a subject suffering from endotoxemia and a suitable carrier.

The invention additionally provides a composition for the treatment of a subject suffering from endotoxin-related anemia comprising purified BPI or a biologically active polypeptide analog thereof in an amount effective to treat a subject suffering from endotoxin-related anemia and a suitable carrier.

Additionally, the invention provides a composition for the treatment of a subject suffering from endotoxin-related leukopenia. The composition comprises purified BPI or a biologically active polypeptide analog thereof in an amount effective to treat a subject suffering from endotoxin-related leukopenia and a suitable carrier. Further provided is a composition for the treatment of a subject suffering from endotoxin-related thrombocytopenia. The composition comprises purified BPI or a biologically active polypeptide analog thereof in an amount effective to treat a subject suffering from endotoxin-related thrombocytopenia and a suitable carrier.

Also, the invention provides a composition for inhibiting lipopolysaccharide-mediated tumor necrosis factor production by cells comprising purified BPI or a biologically active polypeptide analog thereof in an amount effective to inhibit lipopolysaccharide-mediated tumor necrosis factor production by cells and a suitable carrier. Moreover, the invention provides a composition for inhibiting gram negative bacteria-mediated tumor necrosis factor production by cells comprising purified BPI or a biologically active polypeptide analog thereof in an amount effective to inhibit gram negative bacteria-mediated tumor necrosis factor production by cells and a suitable carrier.

The invention provides a composition for inhibiting a pyrogen. The composition comprises purified BPI or a biologically active polypeptide analog thereof in an amount effective to inhibit a pyrogen.

Additionally, the invention provides a method of preventing a condition associated with a gram negative bacterial infection in a subject which comprises administering to the subject an amount of Bactericidal/Permeability Increasing Protein effective to prevent the gram negative bacterial infection and thereby prevent the condition. In the previously described method the condition is any of the conditions selected from the group consisting of endotoxin-related shock, endotoxemia, endotoxin-related anemia, endotoxin-related leukopenia, or endotoxin-related thrombocytopenia.

Moreover, also provided is a method of preventing a disorder involving disseminated intravascular coagulation in a subject which comprises administering to the subject an amount of Bactericidal/Permeability Increasing Protein effective to prevent the symptoms of disseminated intravascular coagulation and thereby preventing the disorder.

Finally, the invention provides a method of isolating and recovering purified Bactericidal/Permeability Increasing Protein which comprises: (a) obtaining a crude sample of Bactericidal/Permeability Increasing Protein; and (b) separating the crude sample by column chromatography using de-pyrogenated solutions thereby isolating and recovering purified Bactericidal/Permeability Increasing Protein. Moreover, in this method the Bactericidal/Permeability Increasing Protein comprises native Bactericidal/Permeability Increasing Protein or a biologically active polypeptide analog thereof. Further, the biologically active polypeptide analog of Bactericidal/Permeability Increasing Protein comprises a polypeptide which has a molecular weight of about 25 kD and corresponds to the N-terminal amino acid sequence of Bactericidal/Permeability Increasing Protein. We have found that biological activity level of BPI varies depending on the method used for obtaining BPI. It appears that depyrogenated BPI, i.e. BPI isolated and recovered by the above-described method using de-pyrogenated solutions, shows a much higher level of biological activity than pyrogen-containing BPI (Table 5 and Figure 4A).

This invention is illustrated in the Experimental Details and Results sections which follow. These sections are set forth to aid in an understanding of the invention but are not intended to, and should not be construed to, limit in any way the invention as set forth in the claims which follow.

Experimental Details

Example 1

Materials and Methods:

Reagents

Lipopolysaccharide from E. Coli 0111:B4, S. typhimurium wild type, glycolipid from S. typhimurium RE mutant, and Lipid A from S. typhimurium RE mutant, and LPS from P. aeruginosa were purchased from RIBI Immmunochem Research, Inc., Hamilton, MT; Fmet-Leu-Phe (FMLP) and polymyxin B Sulfate from Sigma Chemical Co., St. Louis, MO; Hank's Balanced Salt Solution without calcium, magnesium and phenol red (HBSS) from Hazelton Research Products, Denver, PA; Ficoll-Paque, Percoll and Macrodex from Pharmacia Inc., Piscataway, NJ; TNF and anti-TNF from Endogen, Boston MA; Fluorescein conjugated goat-anti-mouse IgG from TAGO Inc., Burlingame, CA: IgG1 control antibody from Coulter Immunology, Hialeah, FL; Phycoerythrin (PE) conjugated anti CR3 (Leu-15) and IgG2a control from Becton Dickinson, Mountain View, CA, Anti CR1 monoclonal antibody, Yz-1, was a kind gift from Dr. Rick Jack at Harvard University

Azurophil Granule Isolation and Extraction

Granulocytes were isolated from buffy coats obtained from local blood banks. Buffy coats were diluted 3-4X in HBSS and granulocytes were separated from mononuclear cells by centrifugation through 64% Percoll. The pellet was subjected to diisopropylfluorophosphate (DFP), washed, and resuspended in ice

cold lysis buffer (10 mM PIPES, pH 6.8, 100 mM KCL, 3mM NaCl, 3.5 mM MgCl2) and disrupted by nitrogen cavitation (Parr Instrument Co., Moline, IL). Azurophil granules were isolated on discontinuous Percoll gradients as described by Borregaard. [Borregaard, N., J.M. Heiple, E.R. Simons, and R.A. Clark, J. Cell. Biol., 97: 52-61 (1983)] The azurophil granules were collected and Percoll was removed by centrifugation at 180,000 X G for 2 hours. The granules were lysed by 4 cycles of freeze-thaw followed by 1 minute of sonication. The lysed granules were extracted in an equal volume of 100 mM glycine, pH 2 by vortexing intermittently for 1 hour at room temperature. The acid extract was clarified by centrifugation at 30,000 X G for 20 minutes and at 200,000 X G for 30 minutes.

Neutrophil Isolation

Venous blood was drawn from healthy volunteer donors into acid citrate dextrose anticoagulant and immediately placed on ice. Five parts of blood were mixed with 1 part of cold Macrodex, and allowed to settle for 1.5 - 2 hours at 4°C. Leukocyte-rich plasma was washed 1X in cold HBSS, then resuspended in HBSS and layered over Ficoll-Paque. If significant erythrocyte contamination was present, the granulocyte pellet was subjected to hypotonic lysis. The cells were washed 2X in HBSS and resuspended in HBSS + 2% autologous plasma to give a final granulocyte concentration of 1 X 10^6/ml in the incubation mixture.

BPI Purification

Approximately 2 mg of crude azurophil granule extract was separated by size on a Biosil (TSK-250) (7.8 mm x 600 mm) high performance size exclusion column using 50 mM glycine and 100 mM NaC1 buffer, pH 2.0, under isocratic conditions of a flow rate of 1 ml/min. Column fractions with the greatest LPS inhibitory activity contained a large proportion of the 54 KD species as shown by SDS

PAGE. These TSK fractions were pooled and run over an Aquapore weak cation exchange (WCX) column (2.1 mm X 30 mm) using 50 mM citrate, pH 5.5, and eluted in a gradient of 0-75%, of 50 mM citrate and 1 M NaCl (Buffer B) in 25 min, then 75-100% Buffer B in 5 min with a flow rate of 200 ml/min. Material of 57 KD was recovered from cation exchange and appeared as a single band on SDS page. In some experiments BPI was further purified by reverse phase HPLC on a Vydac C4 column loaded for 12 min in 0.1% CH_3CN plus 0.1% TFA, in 30 min with a flow rate of 200 ml/min (Rainin Instruments, Emeryville, CA).

Neutrophil stimulation

Isolated neutrophils were kept on ice until incubated with and without stimuli at 37°C for 30 minutes. Following the incubation, cells were washed in a large volume of cold PBS + 0.05% Na Azide + 2% autologous plasma. Pellets were divided in two, one stained with 50μl control IgG1 antibody (20μg/$1x10^6$ cells), the other with 50μl of 20μg/$1x10^6$ cells anti-CR1 for 30 minutes at 0°C. Following this incubation the cells were washed 2X with PBS + autologous plasma, then stained with goat-anti-mouse IgG-FITC, and in some experiments, 20μl of IgG2a-phycoerythrin (PE) in control wells, and 20μl Leu-15 PE in test wells. Following a 30 minute incubation at 0°C and 2 more washes, the cells were analyzed by flow cytometry on a Becton Dickinson FACStar flow cytometer (Becton Dickinson, Mountain View, CA). Neutrophil stimulation was measured by comparing mean fluorescence intensity of samples which had been incubated in HBSS + 2% autologous plasma alone (control) to those incubated with LPS or LPS which had been preincubated for 30 minutes at 37°C with BPI or polymyxin B. Data are expressed as % stimulation or % inhibition and were calculated using the mean fluorescence intensity (Fl), on a log scale, according to:

% Stimulation = [(Experimental - Control)/ (Maximum - control)] X 100

and

% Inhibition = 1-[(+Inhibitor)-(Control)]/ [(-Inhibitor)-(Control)] X 100.

Amino Acid Analysis

Vapor phase hydrolysis of BPI and amino acid derivitization was performed using a Pico-tag Workstation (Waters, Milford MA) and chromatographic analysis of the phenylthiocarbamyl amino acids was performed on an applied Biosystems 130 A MPLC using Protocols provided by the manufacturer.

Sequence Analysis

BPI N-terminal sequence was analyzed by automated Edman degradation using an applied Biosystems 477A pulse liquid phase sequenator (Applied Biosystems, Foster city, CA). Phenyltheohydantion amino acid analysis was performed on line using an applied biosystems Model 120A liquid chromatograph.

Results

Human neutrophils may be stimulated both in vivo and in vitro by lipopolysaccharide. Upon activation, surface expression of receptors for C3b and C3bi (CR1 and CR3 respectively), increases. Using the Fluorescence Activated Cell Sorter (FACS), fluorescence intensity of freshly isolated human neutrophils was measured following stimulation with increasing doses of 0111:B4 LPS (Figure 1a). Because commonly observed maximum stimulation was at or above 10 ng/ml, experiments testing for inhibition of 0111:B4 LPS used 10 ng/ml as the

stimulatory dose. All experiments were performed in duplicate. In most experiments, data is shown only f r CR1 since we did not observe any condition where neutrophil stimulation caused upregulation of CR1 or CR3 alone (M. Marra et al. (1990) J. Immunol. 144(2):662-666).

To determine whether proteins found in neutrophil azurophil granules could interfere with the neutrophil response to LPS, crude acid extracts of azurophil granules were pre-incubated with LPS for 30 minutes at 37°C. The mixture was then tested for its ability to stimulate neutrophils. Azurophil protein (1 μg/ml) could effectively block stimulation of 1 X 10^6 polymorphonuclear leukocytes (PMN)/ml by 10 ng/ml of LPS (**Figure** 1b). This effect was not observed using glycine extraction buffer preincubated with LPS, nor was there any stimulation of neutrophils using crude extract or glycine buffer control (data not shown).

To further investigate which of the proteins in the extract was/were responsible for inhibitory effect, crude acid extracts were separated by reverse phase HPLC; each peak was assayed separately for LPS inhibitory activity. The identity of each of the peaks was previously determined using a two-dimensional purification approach involving microbore reverse phase HPLC in first dimension followed by SDS PAGE, electroblotting and microsequencing. The azurophil proteins can be

TABLE 1

AZUROPHIL GRANULE-DERIVED PROTEINS

Peak	Identity	1 5 10 15
1	Defensins (HNP-2)	C Y C R I P A C I A G E R R Y
2	Granulocidin (HNP-4)	V C S C R L V F C R R T G L R
3	Eosinophil Cationic Protein (ECP)	X P P Q F T R A Q W F A I Q H
4a	Eosinophil-Derived Neurotoxin (EDN)	K P P Q F T X A Q X F E T Q X
4b	Cathepsin G	I I G G R E S R P H S R P Y M
5a	Lysozyme	K V F E R X E L A R T L K R L
5b	Eosinophil Major Protein (MBP)	T C R Y L L V R S L Q T F S Q
6	Unknown	I V G G R K A R P X Q F P F L
7	Unknown	I V G G H E A Q P H S R P Y M
8a	Myeloperoxidase	V N C E T S C V Q Q P P C F P
8b	Elastase	I V G G R R A R P H A X P F M
9	Bactericidal/Permeability Increasing Protein (BPI)	V N P G V V V R I S Q K G L D

resolved into 10 discrete peaks whose identities are shown in **Table 1**. The amino acid sequences shown are for the first 15 amino acids of the N-terminal.

LPS inhibitory activity of 1μg of each peak is shown in **Figure 2**. As shown, peak 9 had the highest LPS neutralizing activity. The major protein species in this peak has N-terminal identity with Bactericidal/Permeability Increasing Protein (BPI) described previously (Weiss, J., P. Elsbach, I. Olsson and H. Odeberg, J. Biol. Chem, 253 (8): 2664-2672 (1978)). BPI has been shown to contain the majority of the gram negative bactericidal activity in azurophil granule protein extracts. Cathepsin G showed some inhibition of LPS, but the data between experiments were not as reproducible as for peak 9. Cathepsin G has been shown to bind to LPS in vitro and to kill gram negative organisms, although to a lesser extent than BPI. Other proteins which have demonstrated microbicidal activity against gram negative organisms are elastase and the defensins. However, these proteins (1 μg/ml) did not inhibit the stimulatory activity of LPS on neutrophils.

LPS inhibitory activity of crude azurophil extracts was further characterized and purified using size exclusion and ion exchange followed by reverse phase chromatography. LPS inhibitory activity comigrates with a pure 57 KD band seen on SDS PAGE (**Figure 3b**).

Because the buffer used in the RPLC separations [CH_3CN and 0.1% trifluoroacetic acid (TFA)] significantly diminishes the LPS inhibitory activity of BPI (data not shown), and since the material purified from ion exchange chromatography was of high purity as judged by SDS PAGE, size exclusion/ion exchange material was used to generate a dose response curve (**Figure 4a**). Data is shown from two experiments, each performed in duplicate. This size exclusion/ion exchange purified material was confirmed to be BPI by N-terminal sequence analysis. Protein concentration was determined by amino acid analysis.

As seen in **Figure** 4a, about 90 ng/ml of BPI is required for maximal inhibition of the neutrophil response to 10 ng/ml 0111/B4 LPS. The neutrophil response to formulated peptide (10^{-7}M FMLP) was not inhibited by BPI (data not shown).

Figure 4b shows a similar does response curve for the polypeptide antibiotic Polymyxin B (PMB). Polymyxin B binds to the Lipid A moiety of LPS and neutralizes some of its toxic effects both in vivo and in vitro. polymyxin B has been demonstrated to bind to LPS stoichiometrically (Morrison, D.C. and D.M. Jacobs, Immunochem, 13: 813-818 (1976)). The calculated amount of PMB required to inhibit 10 ng/ml of smooth LPS is approximately 0.67 nM. In the subject experiments 0.4 ng/ml, or 0.36 nM of polymyxin B was required to completely inhibit neutrophil stimulation using 10 ng/ml of LPS. 90 ng/ml, or 1.58 nM BPI was required for 100% inhibition of 10 ng/ml LPS.
Therefore, on a molar basis the amount of BPI required to inhibit LPS stimulation of neutrophils in vitro was approximately 4X the amount required for polymyxin B.

To test whether BPI can inhibit LPS from other gram negative organisms, LPS molecules with varying polysaccharide chain lengths and Lipid A were tested in the subject system against 90 ng/ml of 2X purified BPI. Data shown in **Table 2** demonstrates that although the stimulatory dose may vary between these molecules, LPS from both smooth and rough chemotypes as well as Lipid A are all inhibited by BPI.

TABLE 2

LPS	10 NG/ML	1 NG/ML
E.COLI 0111:B4	97	*
S. TYPHIMURIUM WILD TYPE	103	113
S. TYPHIMURIUM RE MUTANT	113	109
S. TYPHIMURIUM RE MUTANT	33	99
LIPID A		
P. AERUGINOSA	112	*

* Low to no stimulation at this endotoxin concentration

EXAMPLE 2

I. PRELIMINARY STUDIES ON NEUTROPHIL BPI

As previously discussed, Bacterial/Permeability-Increasing protein (BPI) is a cationic 50-60,000 m.w. protein first purified from human neutrophil granules by Weiss et al. (Weiss, J., P. Elsbach, I. Olsson and H. Odegerg. 1978. J. Biol. Chem. 253:2664.). BPI alters bacterial cell membrane permeability and has bactericidal activity specifically against gram negative organisms. To date, the literature on BPI has focused exclusively on its bactericidal activity.

We report that BPI binds to LPS and inhibits both neutrophil and monocyte responses to soluble LPS in vitro. BPI also inhibits LPS activity in the Limulus Amebocyte Lysate assay. Our research has identified BPI as a lead molecule for the development of novel therapies against endotoxic shock.

In response to LPS, human neutrophils upregulate cell surface expression of complement receptors CR1 and CR3 (Figures 1a and 5b). To measure this neutrophil response to LPS, we incubated freshly isolated human neutrophils with E. Coli 0111:B4 LPS (Figure 4a), and showed that maximal CR1 upregulation is observed using 10 ng/ml LPS (Figure 4). Neutrophil stimulation with LPS was not inhibited by exogenous anti-TNF antibodies, suggesting that LPS acted directly on neutrophils in this system.

BPI inhibits the neutrophil response to LPS (Figure 4a). Inhibition of CR upregulation was complete at a dose of approximately 1.8-3.6 nM (100-200 ng/ml) BPI compared to 0.4 nM polymyxin B required to inhibit 10 ng/ml smooth LPS (approximate m.w. 15,000) is about 0.7 nM, matching closely with the observed

value of 0.4 nM. On a molar basis, the amount f BPI required to inhibit LPS was approximately 5-fold greater than the amount required for polymyxin B.

BPI inhibits LPS-mediated neutrophil stimulation but not stimulation by either FMLP or TNF (Table 3). These data demonstrate that BPI inhibits LPS directly and does not disrupt neutrophil mechanisms involved in CR upregulation.

Neutralization of LPS by BPI occurred rapidly. Even without preincubation, both BPI (and polymyxin B) inhibited more than 70% of the neutrophil response to LPS (Figure 6). Maximal inhibition was seen following only 5 minutes of preincubation.

BPI inhibits CR upregulation stimulated by LPS from smooth and rough bacterial strains, as well as lipid A (Table 4) Because of the broad range of BPI activity against these different forms of LPS, among which only lipid A and 2-keto-3-deoxy-octonate are shared determinants, it is likely that LPS inhibition by BPI is affected through lipid A.

BPI inhibits other LPS-mediated activities. At a concentration of approximately 9 nM, or 500 ng/ml, BPI significantly inhibited LPS activity in the LAL assay (Figure 7). When LPS and BPI were added together without preincubation no inhibition was observed (data not shown), indicating that BPI acted on LPS, and had no effect on the LAL assay system. BPI also inhibits LPS-mediated TNF production by human adherent mononuclear cells (Table 5).

TABLE 3

Effect of BPI on Neutrophil Stimulation by Various Agents

Inhibition of CR Upregulation on Neutrophils

Stimulus	Dose	% Inhibition CR1	% Inhibition CR3
LPS	10 ng/ml	109	102
FMLP	10^{-7} M	9	11
rTNF	50 U/ml	0	0

Neutrophils were incubated with E. Coli 0111:B4 LPS, FMLP or TNF preincubated in the presence or absence of 2.7 nM BPI. Data is reported as percent inhibition of CR expression in response to each stimulus preincubated with buffer alone.

TABLE 4

Inhibition of LPS and Lipid A induced Neutrophil Stimulation by BPI

Inhibition of CR Upregulation on Neutrophils

Stimulus	Dose (ng/ml)	CR1 % Inhibition	CR3 % Inhibition
None	-	0	0
E. Coli 011:B4 LPS	10	100	99
S. typhimurium Wild Type LPS	10	104	100
S. typhimurium RE Mutant LPS	1	97	95
S. typhimurium RE Mutant Lipid A	1	111	104

Neutrophils were stimulated with LPS and lipid A preincubated with and without 2.7 nM purified BPI. Results are expressed as percent inhibition of fluorescence intensity observed with each type of LPS alone.

TABLE 5

BPI Inhibits LPS-Induced
TNF Production by Human Monocytes

TNF (pg/ml) Produced in Response to LPS Preincubated With*:

LPS (ng/ml)	Medium Alone	100 ng/ml Polymyxin B	500 ng/ml BPI	250 ng/ml BPI	Buffer Control
0	0	0	0	0	0
0.1	61	0	0	0	81
1	1051	96	0	0	1090
10	2053	2154	1490	1746	2325

*E. Coli 0111:B4 LPS, was preincubated with BPI or polymyxin B (PMB), than added to adherent peripheral blood mononuclear cells. TNF production was assayed by ELISA.

BPI was first purified by Elsbach and Weiss in 1978. In our initial studies we isolated BPI from azurophil granule extracts in a single step by reverse phase HPLC. Recovery of BPI activity from reverse phase was poor, probably due to the denaturing conditions. Here we show the purification of LPS inhibitory activity using only non-denaturing steps and demonstrate that most of the activity from neutrophils comigrates with BPI. Improvements in the purification have also led to very high specific activity material as will be shown in the following section.

Figures 8-10 show the three chromatographic steps currently employed in our lab. The absorbance is traced by the solid line and LPS inhibitory activity on the dotted lines. Table 6 shows the recovery of activity and protein and the specific activity, as measured in arbitrary LPS neutralizing units (NU). One neutralizing unit is that amount of BPI that inhibits 0.5 E.U. LPS by 50% in the LAL test. A Commassie stained SDS-PAGE gel, of these pools is shown in Figure 11. Analysis of the purified BPI by microbore reverse phase HPLC (Figure 12) identified a single major peak which accounted for 97% of the total protein by integration. Tryptic mapping of BPI allowed us to sequence several major fragments which further confirm the identity of the protein. The full length published sequence for BPI is known (P.W. Gray et al. (1989) J. Biol. Chem. 264(16):9505).

II. PURIFICATION OF BPI UNDER RIGOROUSLY PYROGEN-FREE CONDITIONS

Materials and Methods

<u>Reagents</u> USP grade sterile irrigation water was obtained from Travenol Laboratories Inc., Deerfield IL; Pyrosart filters from Sartorius GmbH, W. Germany; CM Sepharose FF from Pharmacia, Upsala, Sweden; Polyaspartamide weak cation exchange HPLC column (100 X 4.6mm) from the Nest Group, Southborough MA; Glycine and Bio-Sil G250 size exclusion HPLC column (600 X 7.5mm) from Bio-Rad Laboratories, Richmond CA; Polyacrylamide electrophoresis gels from Novex, Encitas CA; Sequencing and amino acid analysis reagents and buffers from Applied Biosystems Inc., Foster City, CA; Trifluoroacetic acid, constant boiling HCL, hydrolyzate amino acid standard, and BCA protein assay reagents from Peirce Chemical Co., Rockford, IL; Limulus Amebocyte Lysate assay from Whittaker Bioproducts, Inc., Walkersville, MD; Lipopolysaccharide from RIBI Immunochem Research, Inc., Hamilton, MT; HPLC grade Acetonitrile from J.T. Baker, Phillipsburg, NJ; all other buffers and salts used were reagent grade. 18 megohm purity water was prepared by Lab Five ultrapure water system from Technic, Seattle, WA. 0.5M NaOH for sanitization was prepared from reagent grade NaOH pellets and USP water.

<u>Granule extracts from Neutrophils:</u> were prepared as described (U.S. Serial No. 199,206, filed May 26, 1988) except that the percoll separation of azurophil granules was omitted. Instead, whole granule fractions were obtained by centrifuging the post nuclear supernatant at 17,000 g for 20 minutes. The granule pellet was then suspended in a volume of 1 ml of 50 mM glycine pH 2 for every 4X10E8 cells lysed. Resuspended granules were lysed by five freeze/thaw cycles on dry ice ethanol followed by vigorous agitation for one hour at 4 degrees C.

The soluble extract was btained by centrifugation at 30,000g for 30 minutes.

Limulus Amebocyte Lysate assay: was performed as directed by the manufacturer. Where necessary the pH of samples was adjusted to neutrality by the addition of pyrogen free 0.5M phosphate buffer pH 7.4. and salinity was decreased to <150 mM by dilution with USP water.

LPS neutralization assay: was performed as previously described (M. Marra et al. (1990) J. Immunol. 144(2):662-666).

High salt fractionation of granule extracts: 200 mgs of extracted protein were pooled from various preparations and kept on ice. 1 volume of sterile 5M NaCl was added for every 4 volumes of extract. The resulting precipitate was pelleted by centrifugation at 20,000 g for 20 minutes at 4°C. This supernatant was prepared for CM sepharose chromatography by diluting with 4 volumes of USP irrigation water and adjusting the pH with enough 1M Tris pH 7.4 to give a final concentration of 50 mM. Only fresh, sterile, pyrogen free stock salts and buffers were used.

CM Sepharose chromatography: An XK-16 column (Pharmacia) was packed with sufficient resin to give a bed volume of 5mls. The column was installed on a gradient FPLC equipped with a Pl pump for sample loading. Prior to use, all surfaces in contact with the mobile phase were extensively washed with 0.5M NaOH. The column was sanitized by washing at 0.2 mls/min. with 0.5M NaOH for 4 hrs. The column was then re-equilibrated and a blank run was performed. Fractions from the blank run and eluents were tested by LAL assay for pyrogenicity. Prepared extract was loaded at a flow rate of 400 mls/hr. Once loaded the column was washed with 2 to 3 column volumes of starting buffer. The granule extract was kept on ice during loading. The column was run at room

temperature.

Weak cation exchange HPLC: was performed using an Eldex ternary gradient pump equipped with a Rheodyne injector and a Gilson model 111B U.V. detector. Wettable surfaces were washed with 0.5M NaOH followed by extensive rinsing with USP water to remove all traces of base prior to installing the column. Blank fractions and eluents were tested for pyrogenicity as above.

Gel permeation HPLC: was performed with the same precautions and equipment outlined for weak cation exchange HPLC.

Polyacrylamide gel electrophoresis: 8 to 16% acrylamide gradient gels were purchased from Novex and run according to the manufacturers specifications.

Protein sequence determination: An Applied Biosystems 477A pulsed liquid phase sequenator equipped with a 120A PTH amino acid analyzer was used for automated edmund degradation.

Microbore reverse phase HPLC: Material for protein sequencing was prepared by desalting on a 30 X 2.1 mm Aquapore butyl column. The gradient used was 30 to 100% B in 30 minutes at a flow rate of 200 ml/minute. Detector settings were 214 nm wavelength at 2.0 absorbance units full scale (see insert figure X). An HP 3396A was used to integrate and plot data.

Amino Acid Analysis: was performed on the system described above using the PTC column, buffers and separation conditions provided by ABI. Sample hydrolysis and PTC derivatives were prepared using a Pico-Tag workstation from the Waters chromatography division of Millipore using the manufacturer's protocols.

Protein assays: Protein concentrations were determined using BCA method instructions 23230, 23225 fr m Peirce Chemical C . In order to minimize buffer interference, samples wer diluted 10 fold and the micro reagent protocol was used.

RESULTS

BPI purified from azurophil granules was previously shown to inhibit neutrophil activation by LPS and to inhibit LPS directly in the LAL assay. In order to further define the role of BPI and investigate the presence of other similar molecules in both azurophil and specific granules, we undertook the purification of LPS inhibitory activity from whole granules extracted at acid pH. Preliminary studies verified the presence of LPS inhibitory activity in the crude extract.

To identify the endotoxin neutralizing activity we attempted its purification from whole granule extracts. Purification of LPS neutralizing activity was greatly enhanced by the observation that high concentrations of NaCl (1M) caused the reversible precipitation of about ninety percent of the protein present in the granule extract. Essentially all of the LPS inhibitory activity remained in the soluble supernatant. The soluble fraction was then diluted, to reduce the ionic strength, and further purified and concentrated by CM sepharose cation exchange chromatography. A broad peak of activity eluted which was subsequently further purified using a polyaspartamide high performance cation exchange column. A somewhat sharper peak of activity was recovered which comigrated with a major protein of about 55,000 molecular weight by SDS-PAGE along with several lower molecular weight proteins. Gel permeation HPLC was used as the final purification step and identified a peak of activity which eluted with a single sharp protein peak. The purified protein migrated as two closely spaced bands on SDS-PAGE at 55,000 molecular weight. 25% of the total endotoxin neutralizing

activity was recovered with a 250 fold purification.

The purified endotoxin neutralizing pr tein was subjected to r verse phase HPLC followed by N-terminal sequence analysis by automated Edman degradation. The sequence, shown in figure 6 was identified as bacterial permeability increasing protein by virtue of complete homology through 39 residues. In addition the amino acid composition of the purified molecule was virtually identical to that of BPI (data not shown).

To investigate whether both closely spaced bands were BPI we subjected the purified proteins to western blotting analysis using BPI-specific rabbit polyclonal antisera raised against a synthetic peptide comprising amino acids 1-20 of BPI. Both bands were immunoreactive. The differences may arise from glycoslyation.

III. LPS INHIBITORY ACTIVITIES OF BPI IN VITRO

Purification of BPI under rigorously pyrogen-free conditions, as described in section II resulted in a more potent BPI preparation as shown by the dose

TABLE 6

	Recovery		Specific
	Activity	Protein	Activity
Extract	100%	100%	0.11 NU/µg
Precipitated	149%	17.3%	0.93 NU/µg
Step 1	35%	1.50	2.51 NU/µg
Step 2	14%	0.75	1.97 NU/µg
Step 3	18%	0.10%	18.9 NU/µg

response curve in Figure 13. Inhibition of LPS-mediated CR upregulation was complete at 25 ng/ml BPI, representing a 4-fold increase in activity compared to the material used in section I. On a molar basis this BPI preparation inhibited LPS at approximately stoichiometric proportions, equivalent to molar inhibitory concentrations of polymyxin B. BPI also inhibited LPS-mediated TNF production by human adherent mononuclear cells at a lower concentration following purification under pyrogen-free conditions (Tables 7 and 8).

BPI binds to LPS (Figure 14). In these experiments, 4 ug of LPS/well was immobilized on 96 well plastic plates, then incubated with varying concentrations of BPI, and developed with anti-BPI polyclonal antisera. BPI binding to LPS was inhibited by polymyxin B (Figure 15), demonstrating specificity of BPI binding. BPI binds to LPS in the presence of both plasma (Figure 16) and serum (Figure 17), demonstrating potential in vivo efficacy of BPI.

TABLE 7

BPI Inhibits LPS-Induced TNF Production by Human Monocytes

TNF (pg/ml) Produced in Response to LPS Preincubated with*:

LPS ng/ml	Medium alone	100 ng/ml PMB	400 ng/ml BPI	150 ng/ml BPI	25 ng/ml BPI	Buffer Control
0	0	0	0	0	0	0
0.1	98	79	0	0	0	269
1	1150	1207	0	0	0	1292
10	1370	1270	145	353	559	1413

*E. Coli 0111:B4 LPS, was preincubated with BPI or polymyxin B (PMB), then added to adherent peripheral blood mononuclear cells.
TNF production was assayed by ELISA.

TABLE 8

INHIBITION OF LPS-INDUCED
TNF PRODUCTION BY HUMAN MONOCYTES

TNF (pg/ml) Produced in Response to LPS Preincubated with*:

LPS	1000 ng/ml Polymyxin B	100 ng/ml Polymyxin B	250 ng/ml BPI	50 ng/ml BPI	10ng/ml BPI	Buffer Control
10	333±18	601±257	270±23	270±67	436±38	697±37
100	769±101	1140±73	834±30	686±84	1005±50	892±47
1000	844±144	1016±20	1130±10	778±189	1025±71	723±88
S.aureus						
	1685±121	1541±397	1558±139	1268±374	1554±324	1423±447

*BPI or polymyxin B sulfate were preincubated with 0-10 ng/ml E. Coli 0111:B4 LPS or 0.1%w/v killed S. aureus then added adherent peripheral blood mononuclear cells. TNF production was assayed by ELISA.

EXAMPLE 3

BPI/Endotoxin Pyrogenicity

Stage IA-Pyrogenicity of Glycine Buffer:

305µl of Glycine Buffer control (Supplied by Redwood City) was diluted to 7 ml in PBS (Redwood City) and mixed in polypropylene tubes (pyrogen-free). The tube was labeled with notebook #1990 and tested in a three rabbit USP Rabbit Pyrogen assay at a dose of 2 ml/rabbit (actual injection dose was 2.1 ml/rabbit).

The product was non-pyrogenic; it produced a total temperature rise for all three rabbit of 0.4 C.

Stage IB-Pyrogenicity of 2 ug of BPI:

304 µl of BPI (Lot 78038, dated 8/19/89) was diluted to 7 ml using PBS (Redwood City) and mixed in polypropylene tubes (pyrogen-free). The Tube was labeled with notebook #20170 and tested in a three rabbit USP Pyrogen assay at a dose of 2.0 ml/rabbit.

The product was non-pyrogenic as demonstrated by a total temperature rise of 0.2°C.

Stage II-Pyrogenicity of BPI pre-incubated with endotoxin:

Endotoxin from E. Coli 055.B5 (Sigma Chemicals) was diluted in PBS (Redwood City) to 4096 EU/ml. This concentration was confirmed by the LAL Assay.

304 ul f BPI (Lot 78038, dated 8/19/89) was diluted to 7 ml with the PBS diluted endotoxin (4096 EU/ml) hereinabove using polypropylene tubes. The tube was mixed by vortexing to effect mixing. The BPI+Endotoxin and Endotoxin in PBS were incubated at 37°C in a water bath for 30 minutes. Following incubation at 37°C the BPI+Endotoxin showed an endotoxin concentration of 122 EU/ml. The endotoxin diluted in PBS did not show a change in the end point of 4096 EU/ml.

The BPI+Endotoxin and Endotoxin in PBS in PBS were tested in the three rabbit USP pyrogen assay and were found pyrogenic with total temperature rises of 4.6°C and 7.5°C, respectively.

<u>Stage II (Repeat):</u>

To achieve improved results with the manipulations of the endotoxin preparation we switched from the <u>E. coli</u> 055:B5 from Sigma to the Official FDA References.

A vial of EC-5 was rehydrated with PBS (Redwood City) to 2 ml to give a concentration of 5000 EU/ml. We verified by the label claim of 10,000 EU/ml by LAL assay.

The BPI+Endotoxin sample was prepared by adding 38µl of PBI (Lot 78038) to 7.3 ml of PBS plus 320µl of the 5000 EU/ml of EC-5 endotoxin. The preparation was mixed in a polypropylene tube(pyrogen-freed) and mixed well. An 8.0 ml sample of EC-5 endotoxin was prepared in PBS(Redwood City) to the same concentration without the addition of BPI. Both samples was incubated at 37°C for 30 minutes in a water bath.

The two samples were tested for endotoxin activity using the LAL assay. The BPI+Endotoxin was negative. The endotoxin sample was positive at the target

of 200 EU/ml (Figure 18).

Both samples were tested in the three rabbit USP Pyrogen Assay at a dose of 2.0 ml/rabbit.

The BPI+Endotoxin was non-pyrogenic and caused a total temperature rise of 1.1°C. The EC-5 endotoxin in PBS was pyrogenic and caused a total temperature rise of 3.9°C.

What is claimed is:

1. A method of inhibiting lipopolysaccharide-mediated stimulation of cells which comprises contacting the cells in the presence of a cell-stimulating amount of lipopolysaccharide, with Bactericidal/Permeability Increasing Protein in an amount effective to inhibit cell stimulation.

2. A method of claim 1, wherein the cells are neutrophils or monocytes.

3. A method of claim 1, wherein the amount effective to inhibit cell stimulation comprises an amount from about 100 ng to about 100 mg.

4. A method of claim 3, wherein the amount effective to inhibit cell stimulation comprises an amount from about 10 ug to about 10mg.

5. A method of claim 1, wherein the Bactericidal/Permeability Increasing Protein comprises purified Bactericidal/Permeability Increasing Protein.

6. A method of claim 1, wherein the Bactericidal/Permeability Increasing Protein comprises recombinant Bactericidal/Permeability Increasing Protein or a biologically active polypeptide analog thereof.

7. A method of claim 6, wherein the biologically active polypeptide analog of Bactericidal/Permeability Increasing Protein comprises a polypeptide which has a molecular weight of about 25 kD and corresponds to the N-terminal amino acid sequence of Bactericidal/Permeability Increasing Protein.

8. A method of treating a gram negative bacterial infection which comprises

c ntacting the bacterial infection with purified Bactericidal/Permeability Increasing Protein or a biologically active polypeptide analog thereof in an amount effective to inhibit lipopolysaccharide-mediated stimulation of cells and thereby treat the bacterial infection.

9. A method of claim 8, wherein the cells are neutrophils or monocytes.

10. A method of claim 8, wherein the gram negative bacterial infection is associated with endotoxic shock.

11. A method of claim 8, wherein the gram negative bacterial infection is associated with an inflammatory condition.

12. A method of claim 11, where the inflammatory condition is associated with disseminated intravascular coagulation, adult respiratory distress syndrome, or renal failure.

13. A method of claim 8, wherein the gram negative bacterial infection is present in a subject.

14. A method at claim 13, wherein the subject is a human-being.

15. A composition for the treatment of a gram negative bacterial infection comprising purified Bactericidal/Permeability Increasing Protein or a biologically active polypeptide analog thereof in an amount effective to inhibit lipopolysaccharide-mediated stimulation of cells and a suitable carrier.

16. A pharmaceutical composition of claim 15, wherein the suitable carrier comprises a pharmaceutically acceptable carrier.

17. A method of treating a subject suffering from endotoxin-related shock caused by a gram negative bacterial infection which comprises administering to the subject an amount of Bactericidal/Permeability Increasing Protein effective to combat the gram negative bacterial infection and treat the subject so as to alleviate the endotoxin-related shock.

18. A method of treating a subject suffering from a disorder involving disseminated intravascular coagulation which comprises administering to the subject an amount of Bactericidal/Permeability Increasing Protein effective to alleviate the symptoms of disseminated intravascular coagulation and thereby treat the subject.

19. A method of treating a subject suffering from endotoxin-related anemia caused by a gram negative bacterial infection which comprises administering to the subject an effective amount of Bactericidal/Permeability Increasing Protein effective to combat the gram negative bacterial infection and treat the subject so as to alleviate endotoxin-related anemia.

20. A method of treating a subject suffering from endotoxin-related leukopenia caused by a gram negative bacterial infection which comprises administering to the subject an effective amount of Bactericidal/Permeability Increasing Protein effective to combat the gram negative bacterial infection and treat the subject so as to alleviate endotoxin-related leukopenia.

21. A method of treating a subject suffering from endotoxin-related thrombocytopenia caused by a gram negative bacterial infection which comprises administering to the subject an effective amount of Bactericidal/Permeability Increasing Protein effective to combat the gram negative bacterial infection and treat the subject so as to alleviate endotoxin-related thrombocytopenia.

22. A method of inhibiting a pyrogen which comprises contacting the pyrogen with an amount of Bactericidal/Permeability Increasing protein effective to inhibit the pyrogen.

23. A method of inhibiting lipopolysaccharide-mediated tumor necrosis factor production by cells which comprises contacting the cells in the presence of a cell-stimulating amount of lipopolysaccharide, with Bactericidal/Permeability Increasing Protein in an amount effective to inhibit lipopolysaccharide-mediated tumor necrosis factor production by cells.

24. A method of inhibiting gram negative bacteria-mediated tumor necrosis factor production by cells which comprises contacting the gram negative bacteria with Bactericidal/Permeability Increasing Protein in an amount effective to inhibit gram negative bacteria-mediated tumor necrosis factor production by cells.

25. A method of any of claims 17, 18, 19, 20, 21, 22, 23, or 24, wherein the effective amount of Bactericidal/Permeability Increasing Protein comprises an amount from about 100 ng to about 100 mg.

26. A method of claim 25, wherein the effective amount of

Bactericidal/Permeability Increasing Protein comprises an amount from about 10 ug to about 10 mg.

27. A method of any of claims 17, 18, 19, 20, 21, 22, 23, or 24, wherein the Bactericidal/Permeability Increasing Protein comprises native Bactericidal/Permeability Increasing Protein or a biologically active polypeptide analog thereof.

28. A method of claim 27, wherein the biologically active polypeptide analog of Bactericidal/Permeability Increasing Protein comprises a polypeptide which has a molecular weight of about 25 kD and corresponds to the N-terminal amino acid sequence of Bactericidal/Permeability Increasing Protein.

29. A composition for the treatment of a subject suffering from endotoxin-related shock which comprises purified Bactericidal/Permeability Increasing Protein in an amount effective to treat a subject suffering from endotoxin-related shock and a suitable carrier.

30. A composition for the treatment of a subject suffering from a disorder involving disseminated intravascular coagulation which comprises purified Bactericidal/Permeability Increasing Protein or a biologically active polypeptide analog thereof in an amount effective to treat a subject suffering from the disorder and a suitable carrier.

31. A composition for the treatment of a subject suffering from endotoxin-related anemia comprising purified Bactericidal/Permeability Increasing Protein or a biologically active polypeptide analog thereof in an amount effective to treat a subject suffering from endotoxin-related anemia and a

suitable carrier.

32. A composition for the treatment f a subject suffering fr m endotoxin-related leukopenia comprising purified Bactericidal/Permeability Increasing Protein or a biologically active polypeptide analog thereof in an amount effective to treat a subject suffering from endotoxin-related leukopenia and a suitable carrier.

33. A composition for the treatment of a subject suffering from endotoxin-related thrombocytopenia comprising purified Bactericidal/Permeability Increasing Protein or a biologically active polypeptide analog thereof in an amount effective to treat a subject suffering from endotoxin-related thrombocytopenia and a suitable carrier.

34. A composition for inhibiting lipopolysaccharide-mediated tumor necrosis factor production by cells comprising purified Bactericidal/Permeability Increasing Protein or a biologically active polypeptide analog thereof in an amount effective to inhibit lipopolysaccharide-mediated tumor necrosis factor production by cells and a suitable carrier.

35. A composition for inhibiting gram negative bacteria-mediated tumor necrosis factor production by cells comprising purified Bactericidal/Permeability Increasing Protein or a biologically active polypeptide analog thereof in an amount effective to inhibit gram negative bacteria-mediated tumor necrosis factor production by cells and a suitable carrier.

36. A composition for inhibiting a pyrogen comprising purified Bactericidal/Permeability Increasing Protein or a biologically active

polypeptide analog thereof in an amount effective to inhibit a pyrogen and a suitabl carrier.

37. A method of preventing a condition associated with a gram negative bacterial infection in a subject which comprises administering to the subject an amount of Bactericidal/Permeability Increasing Protein effective to prevent the gram negative bacterial infection and thereby prevent the condition.

38. The method of claim 37, wherein the condition is any of the conditions selected from the group consisting of endotoxin-related shock, endotoxemia, endotoxin-related anemia, endotoxin-related leukopenia, or endotoxin-related thrombocytopenia.

39. A method of preventing a disorder involving disseminated intravascular coagulation in a subject which comprises administering to the subject an amount of Bactericidal/Permeability Increasing Protein effective to prevent the symptoms of disseminated intravascular coagulation and thereby preventing the disorder.

40. A method of isolating and recovering purified Bactericidal/Permeability Increasing Protein which comprises:

(a) obtaining a crude sample of Bactericidal/Permeability Increasing Protein; and

(b) separating the crude sample by column chromatography using de-pyrogenated solutions thereby isolating and recovering purified Bactericidal/Permeability Increasing Protein.

41. A method of claim 40, wherein the Bactericidal/Permeability Increasing

Protein comprises native Bactericidal/Permeability Increasing Protein.

42. A method of claim 40, wherein the Bactericidal/Permeability Increasing Protein comprises a biologically active polypeptide analog thereof.

43. A method of claim 41, wherein the biologically active polypeptide analog of Bactericidal/Permeability Increasing Protein comprises a polypeptide which has a molecular weight of about 25 kD and corresponds to the N-terminal amino acid sequence of Bactericidal/Permeability Increasing Protein.

A. LPS STIMULATION OF GRANULOCYTE CR1

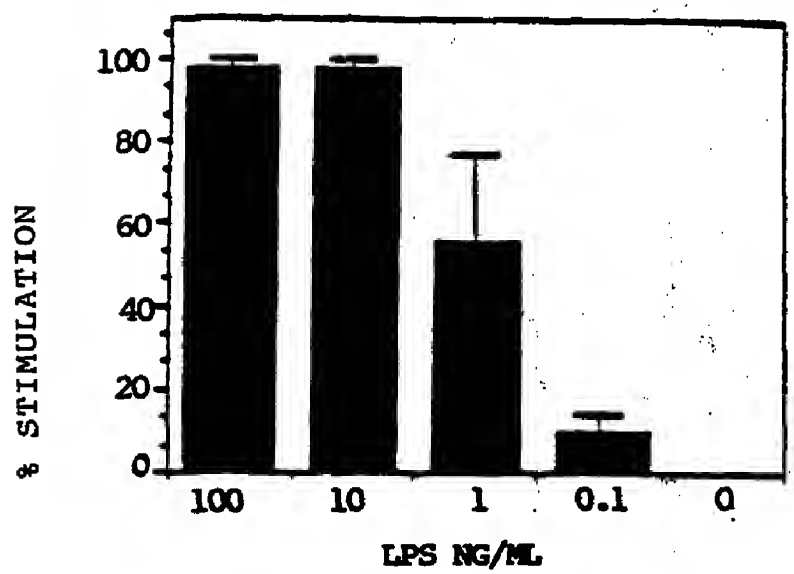


FIGURE 1A

B. NEUTRALIZATION OF LPS BY CRUDE AZUROPHIL EXTRACT

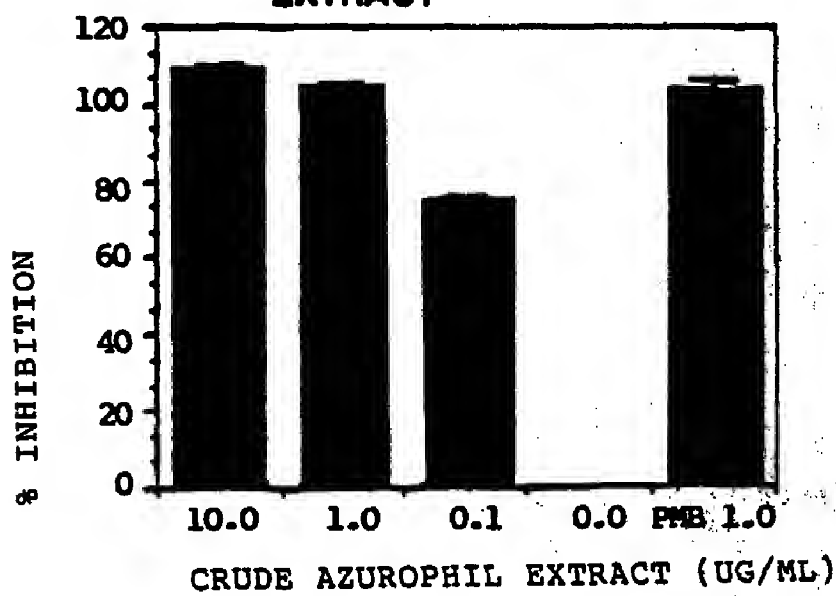


FIGURE 1B

SUBSTITUTE SHEET

FIGURE 2

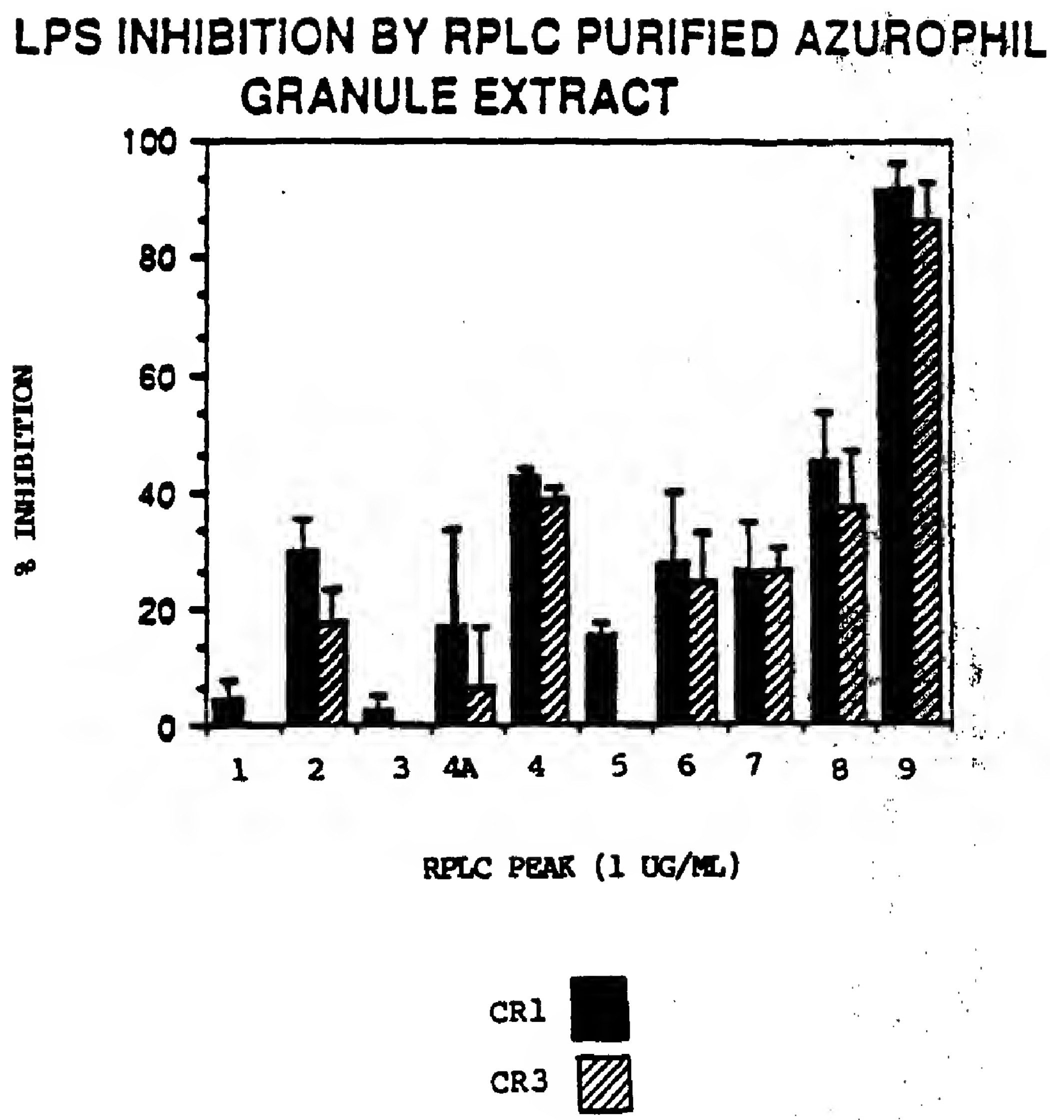

LPS INHIBITION BY RPLC PURIFIED AZUROPHIL GRANULE EXTRACT
% INHIBITION
100
80
60
40
20
0
1
2
3
4A
4
5
6
7
8
9
RPLC PEAK (1 UG/ML)
CR1
CR3

FIGURE 3A

LPS INHIBITORY ACTIVITY OF 3X PURIFIED BPI

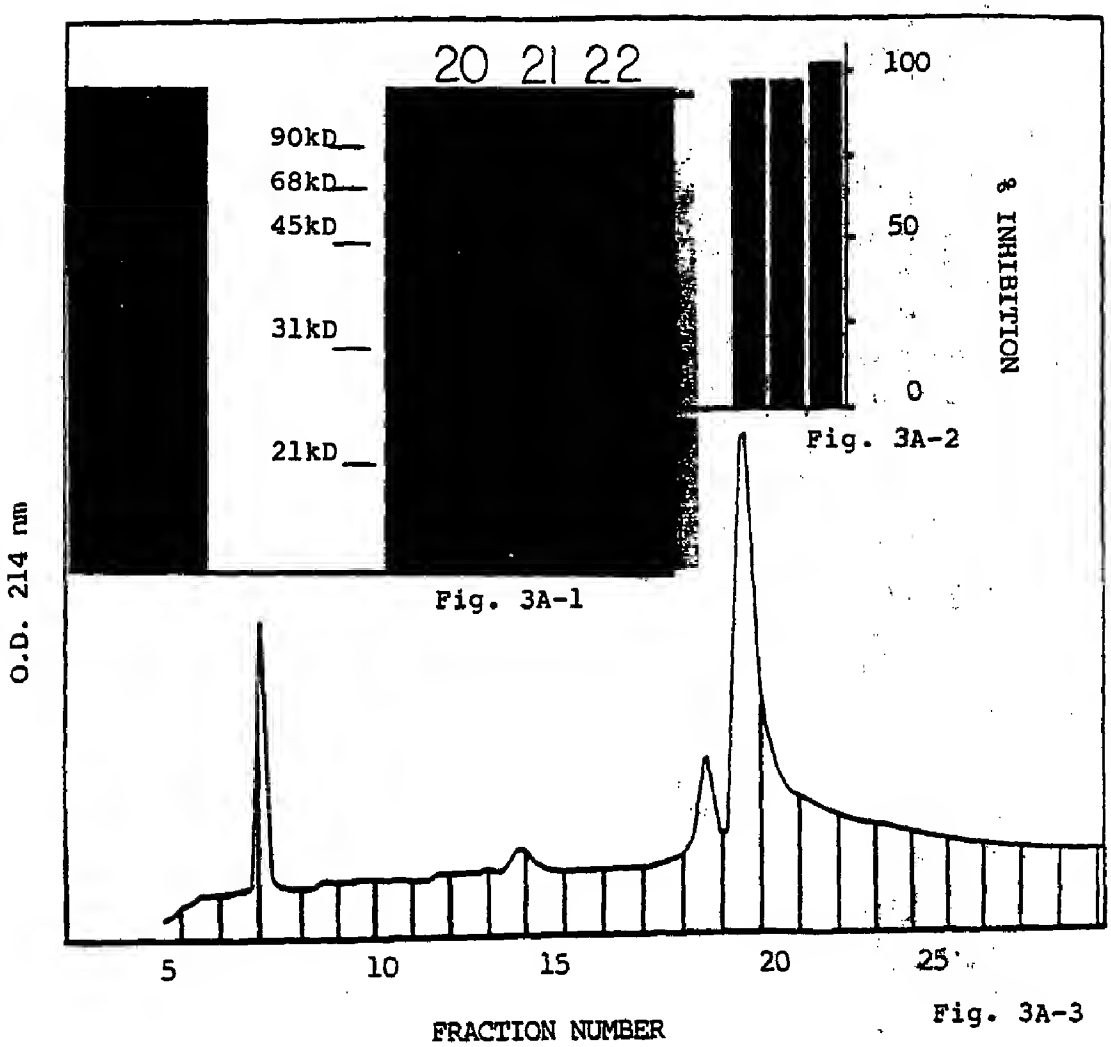


Fig. 3A-1

Fig. 3A-2

Fig. 3A-3

FIGURE 3B

90kD
68kD
45kD
31kD
21kD

A. DOSE RESPONSE OF BPI VS 10 NG/ML 0111:B4 LPS

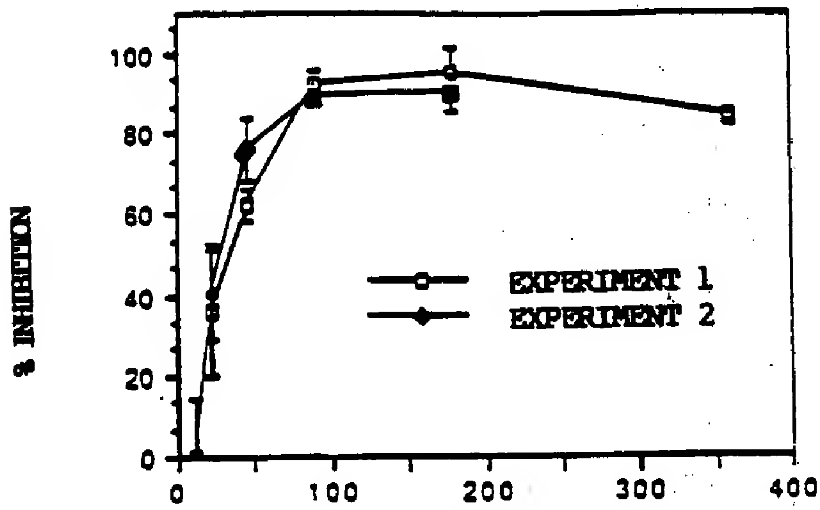


Figure 4a BPI(ng/ml)

B. LPS NEUTRALIZATION BY POLYMIXIN B

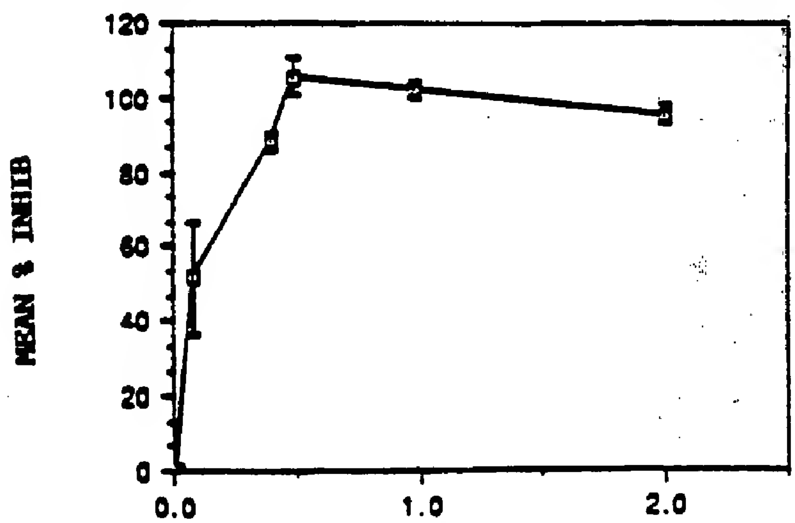
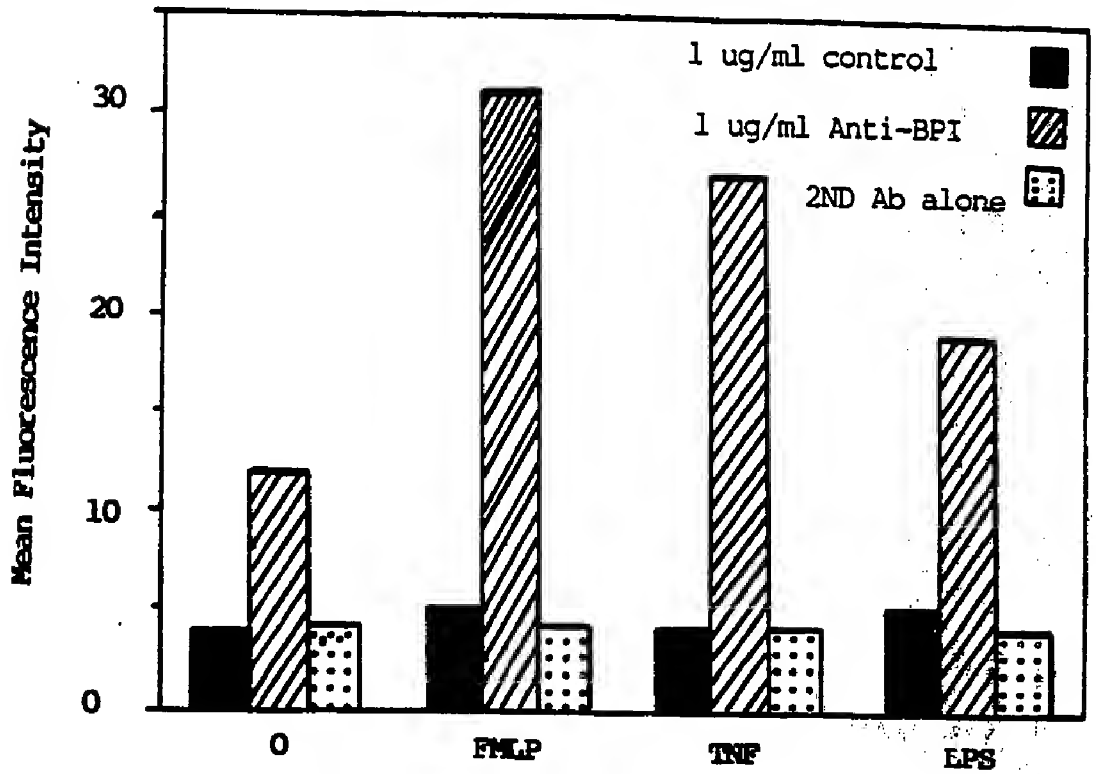


Figure 4b PMS(ng/ml)

FIGURE 5


BPI Expression on the Surface of Stimulated Neutrophils
Mean Fluorescence Intensity
30
20
10
0
1 ug/ml control
1 ug/ml Anti-BPI
2ND Ab alone
O
FMLP
TNF
LPS

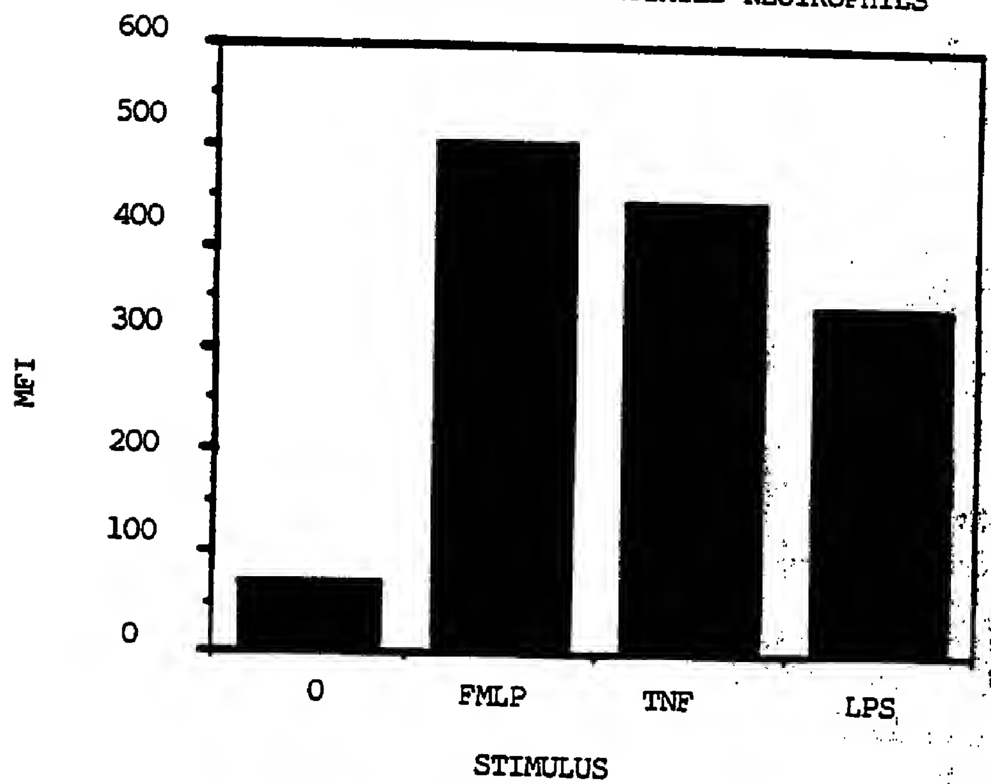
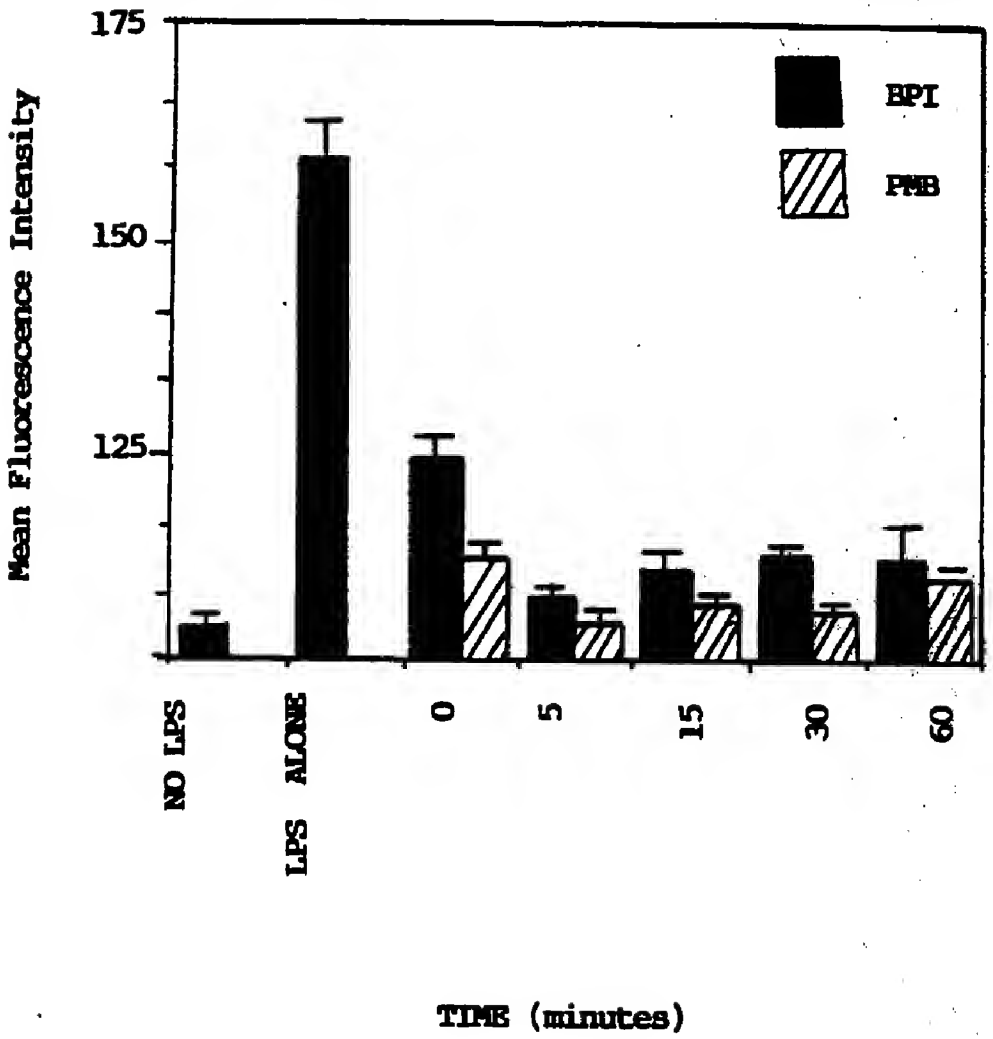

CR3 EXPRESSION ON STIMULATED NEUTROPHILS
600
500
400
300
200
100
0
MFI
0
FMLP
TNF
LPS
STIMULUS

FIGURE 6

Timecourse of LPS Inhibition by BPI and Polymyxin B



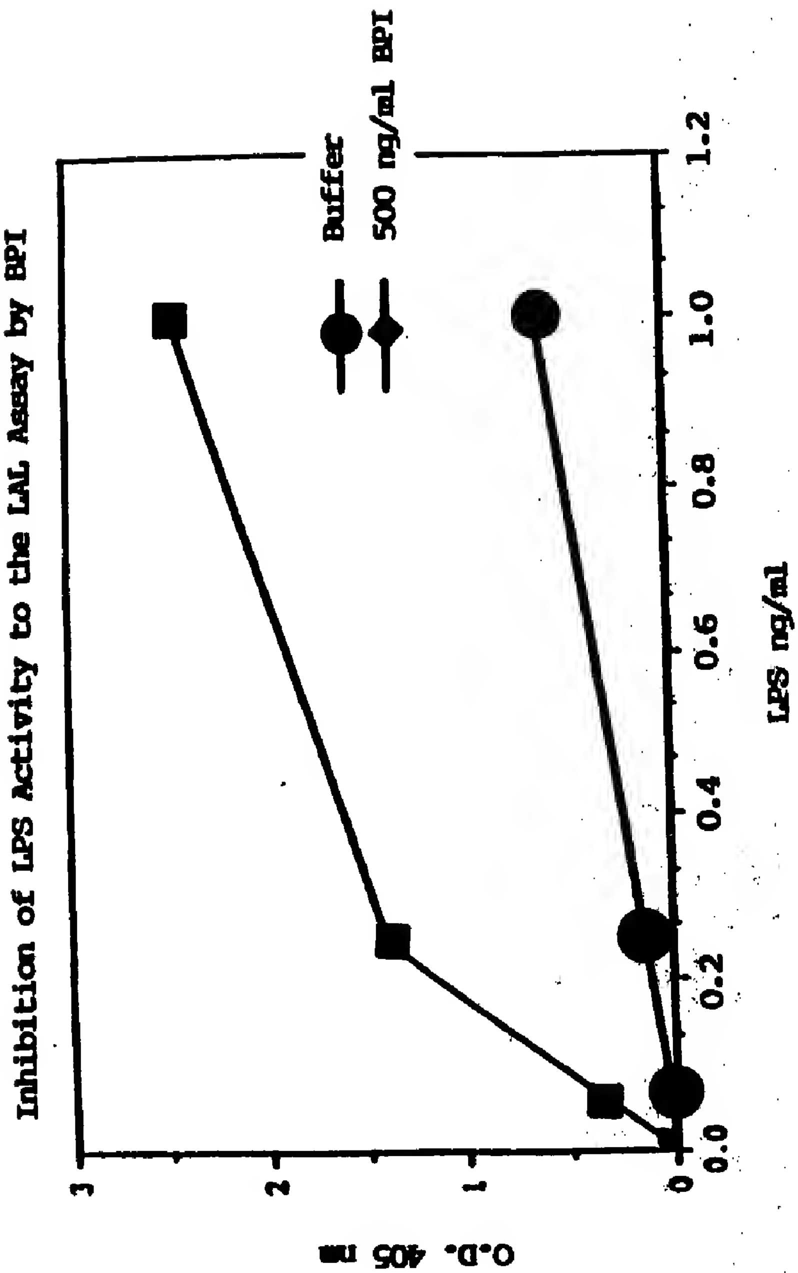

Inhibition of LPS Activity to the LAL Assay by BPI
Buffer
500 ng/ml BPI
O.D. 405 nm
LPS ng/ml
0
1
2
3
0.0
0.2
0.4
0.6
0.8
1.0
1.2

FIGURE 7

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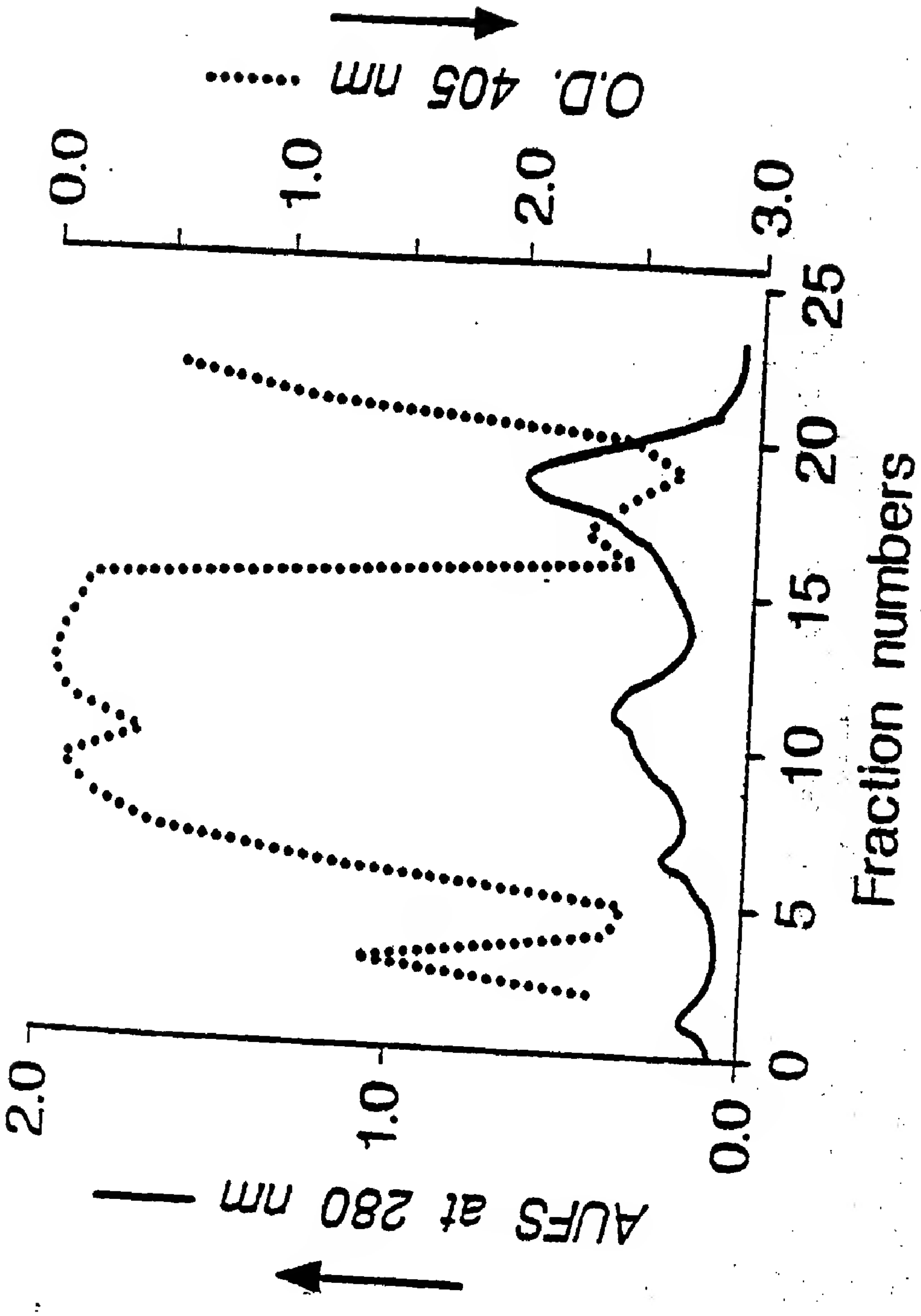
O.D. 405 nm
0.0
1.0
2.0
3.0
AUFS at 280 nm
2.0
1.0
0.0
0
5
10
15
20
25
Fraction numbers

FIGURE 8

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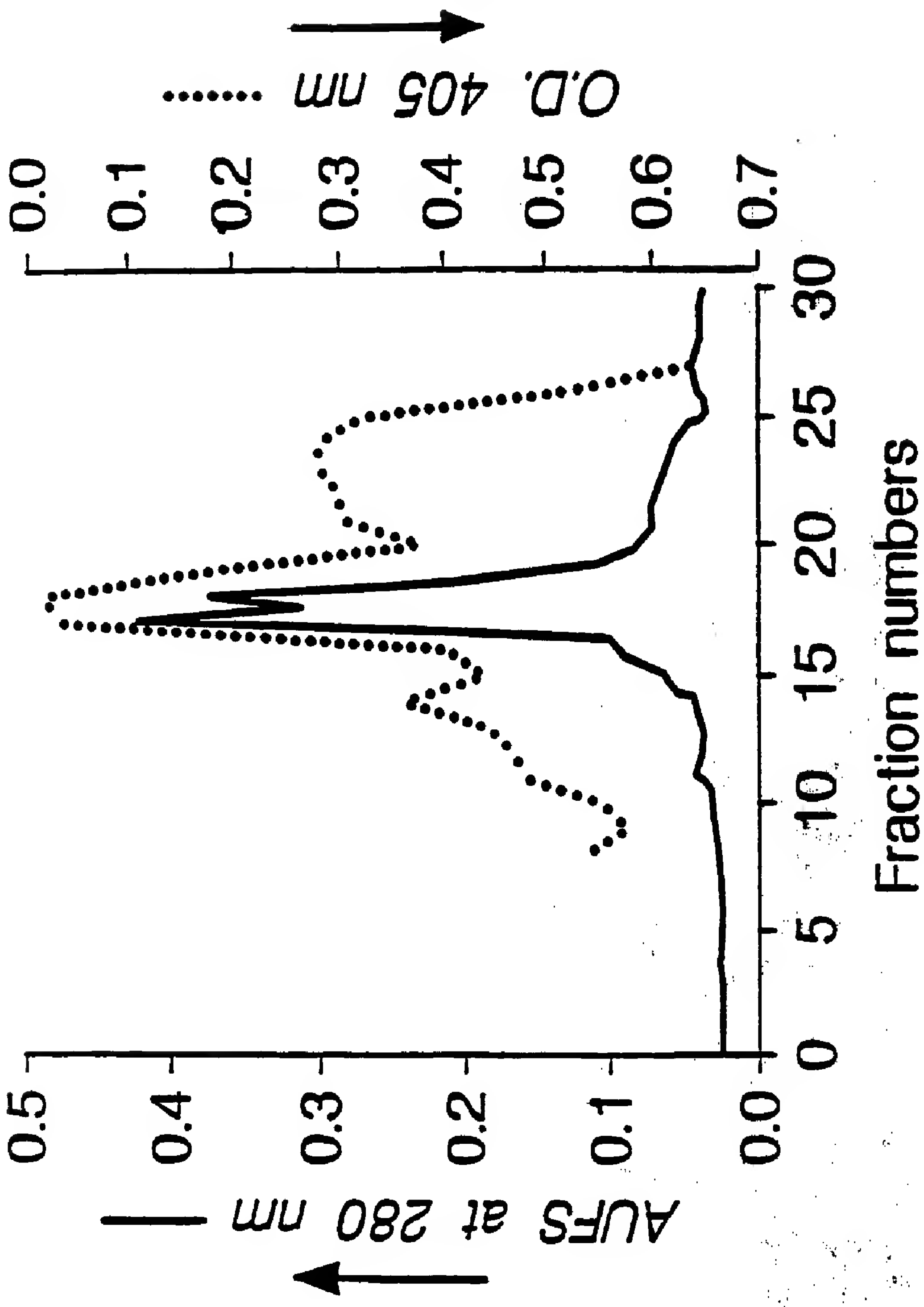


FIGURE 9

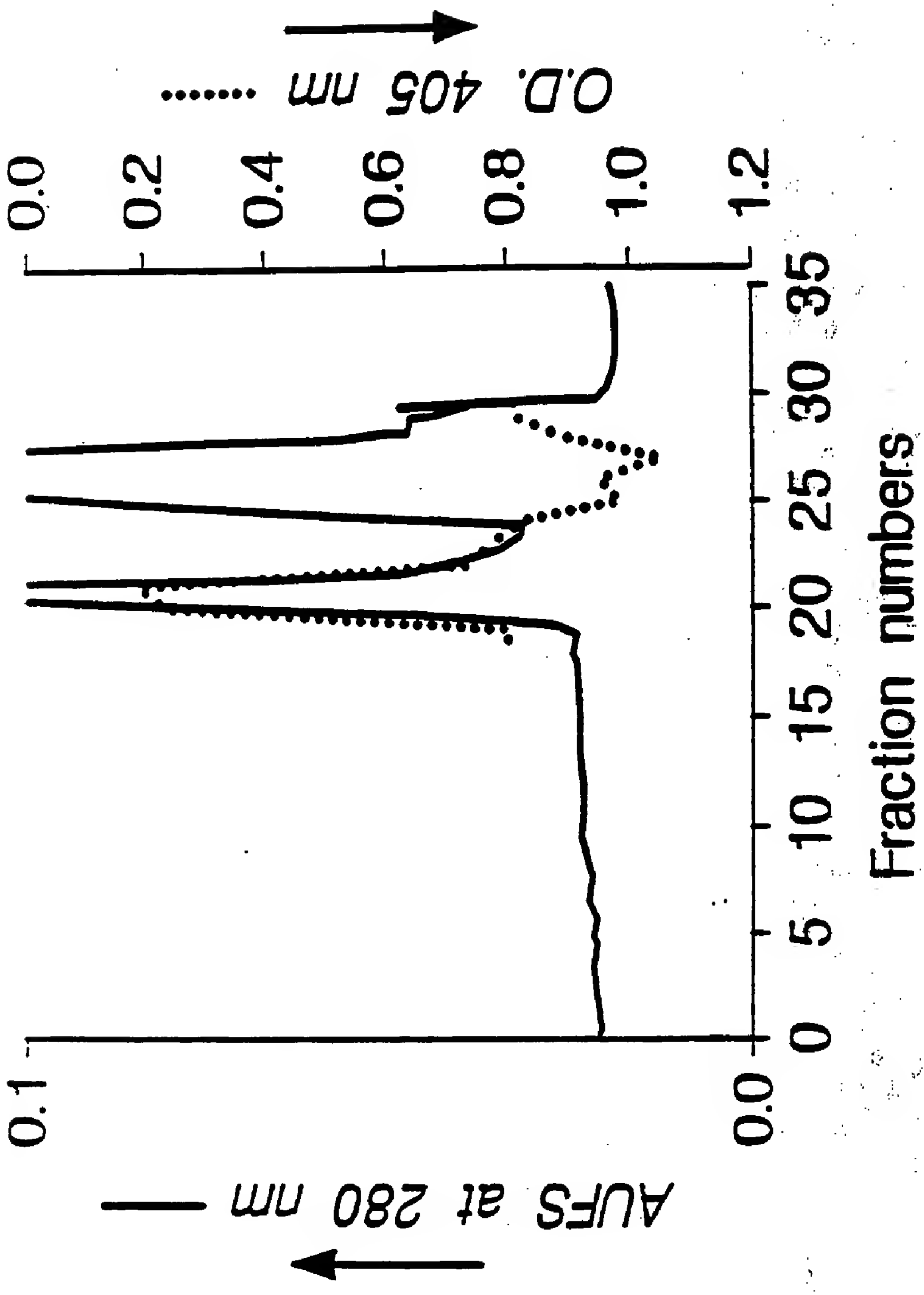

O.D. 405 nm
0.0
0.2
0.4
0.6
0.8
1.0
1.2
AUFS at 280 nm
0.1
0.0
0
5
10
15
20
25
30
35
Fraction numbers

FIGURE 10

FIGURE 11

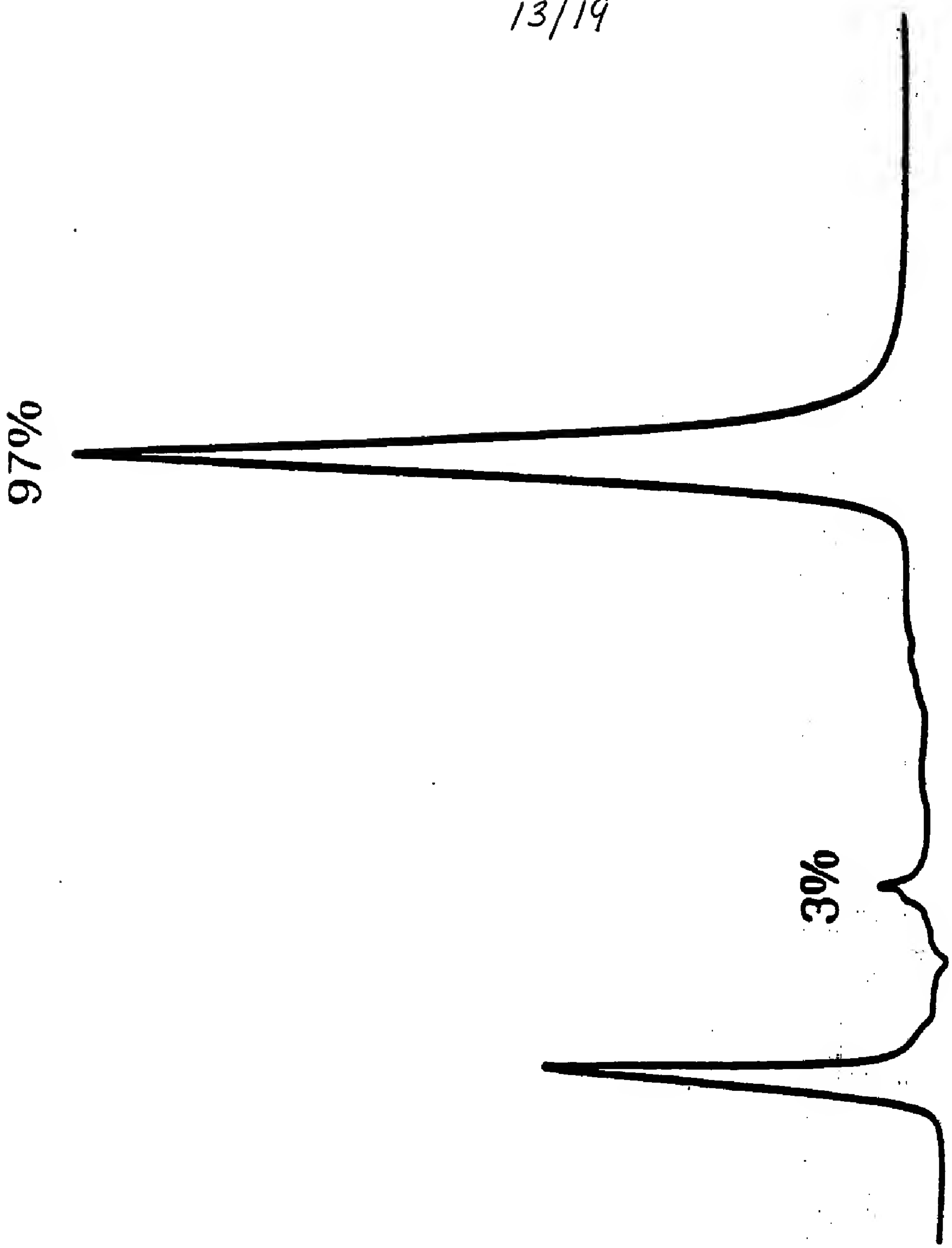


FIGURE 12

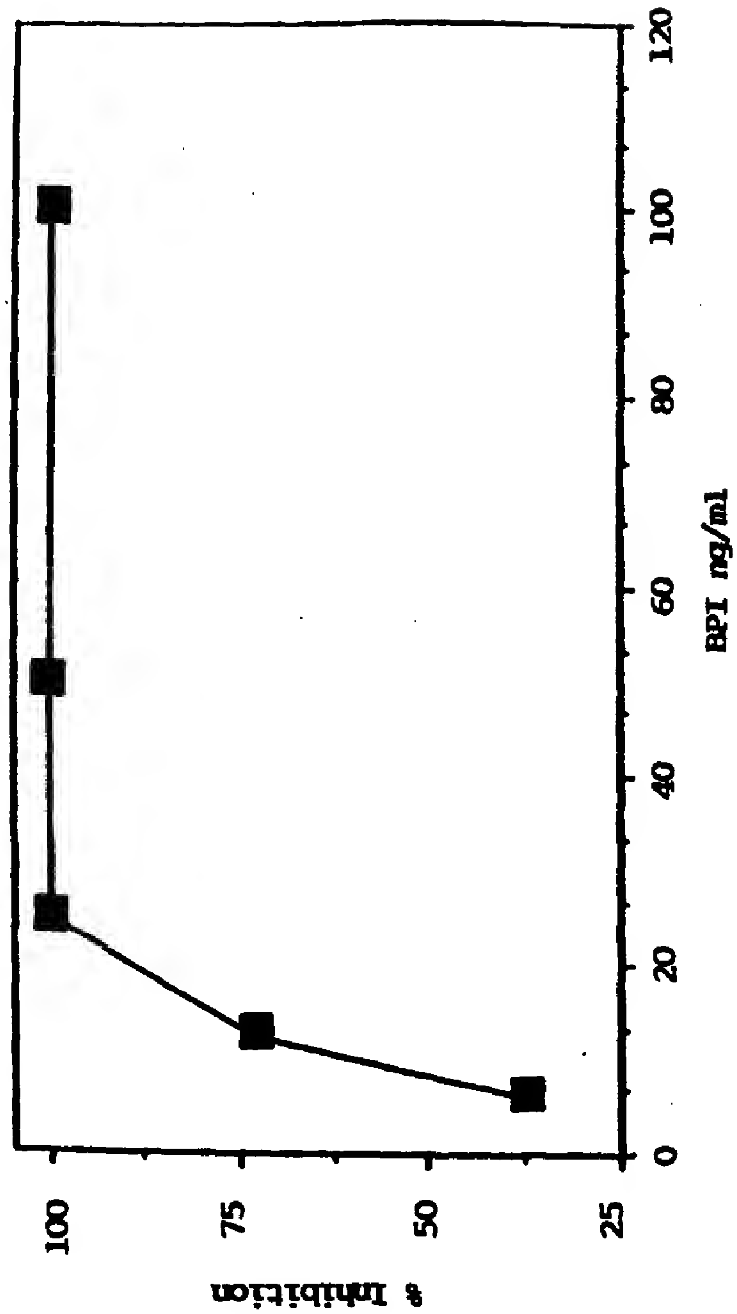
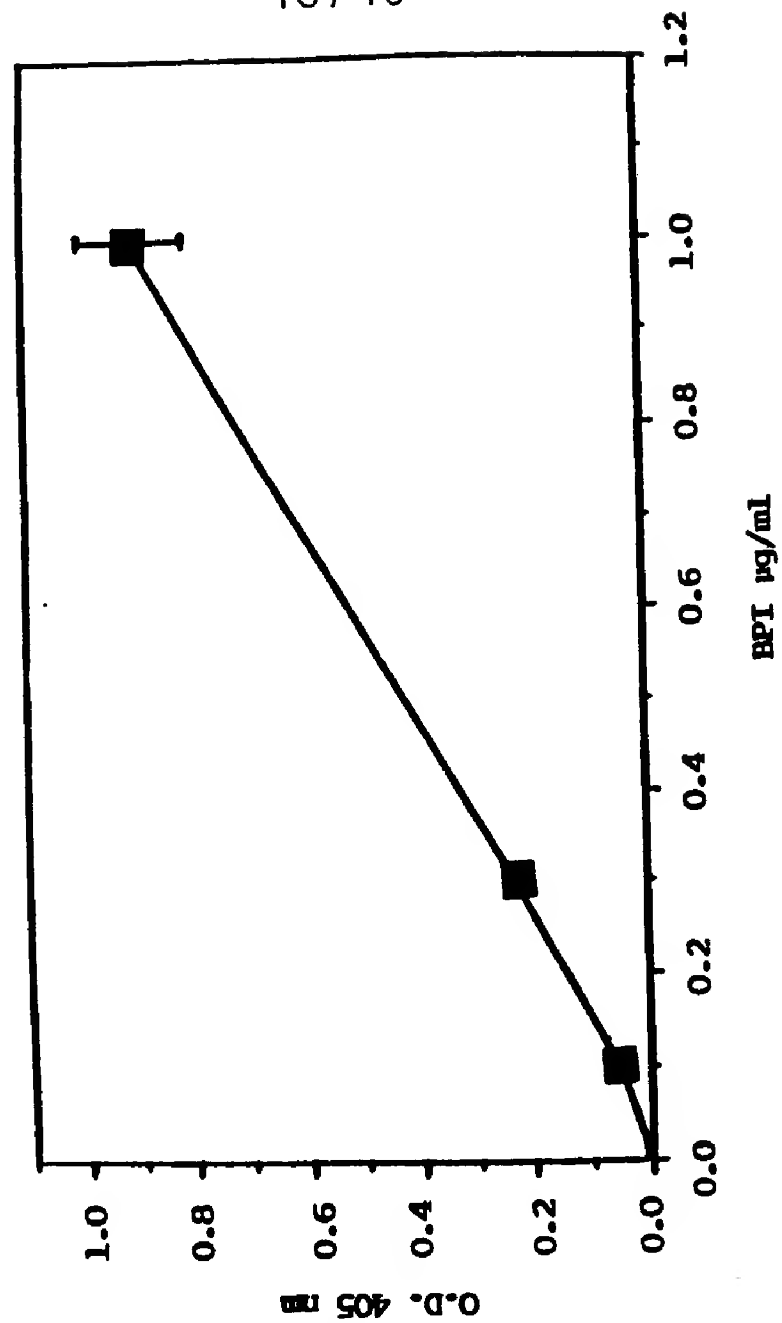


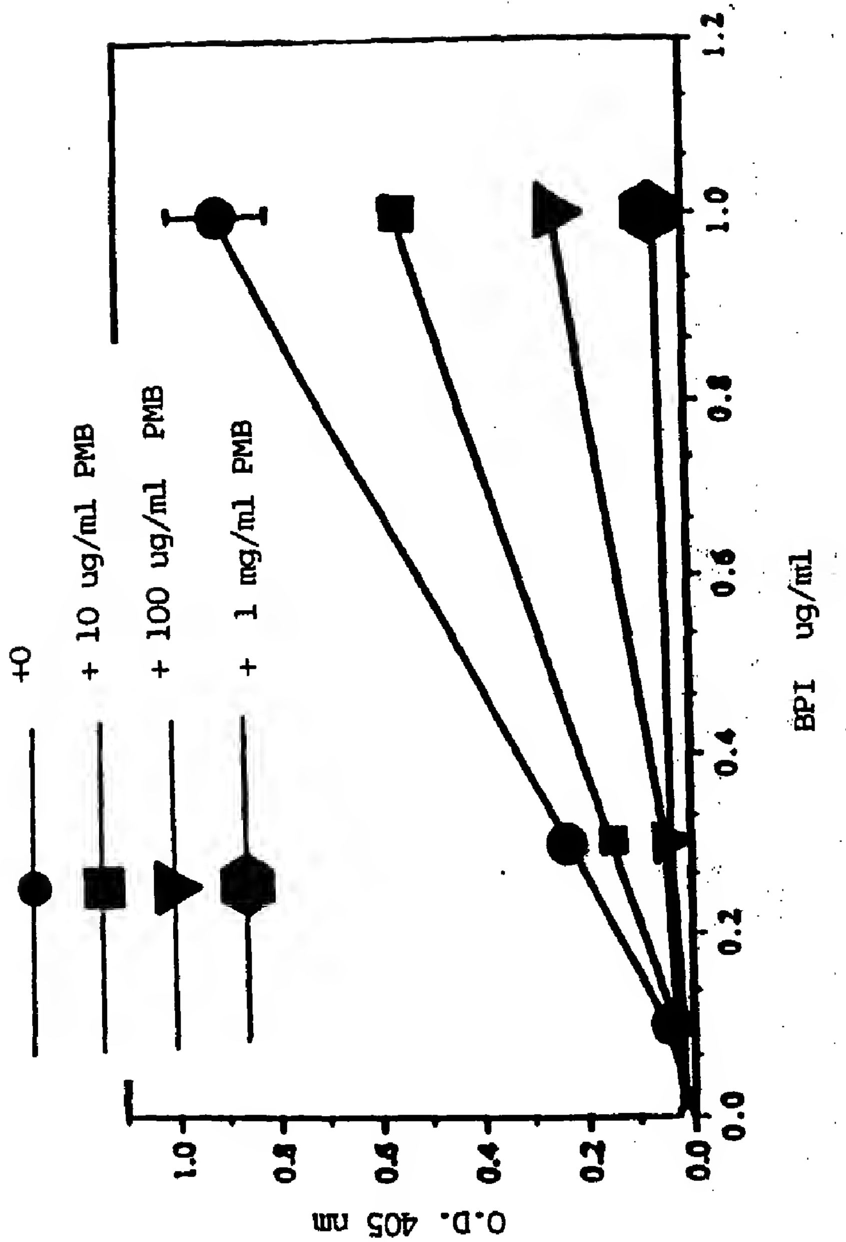
FIGURE 13

Inhibition of the Neutrophil Response to 10 ng/ml LPS by BPI Lot 78052



BPI Binds to LPS Immobilized on Plastic

FIGURE 14



BPI BINDING TO IMMOBILIZED LPS IS INHIBITED BY PMB

FIGURE 15

FIGURE 16

BPI Binds to LPS in the Presence of Plasma

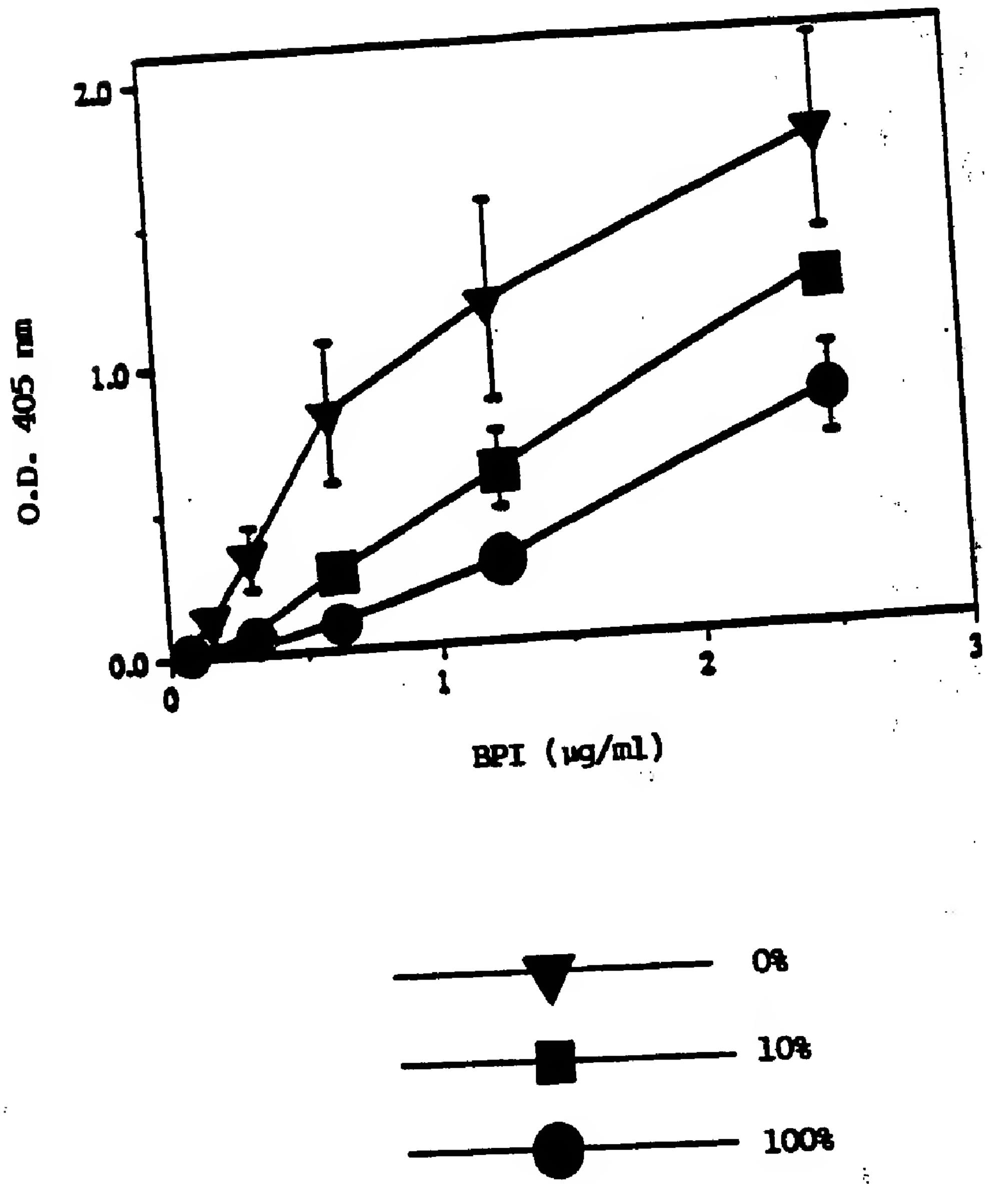
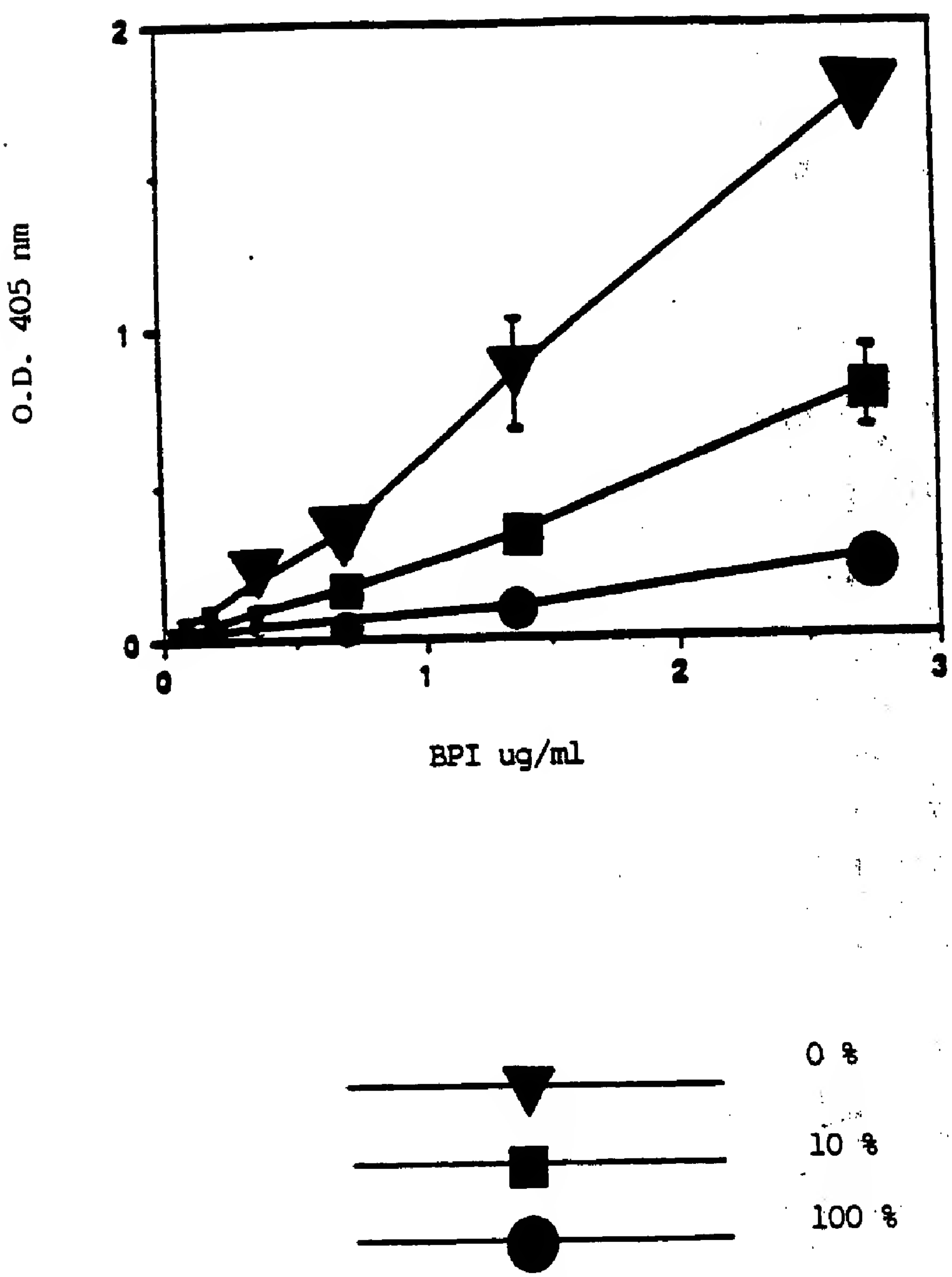


FIGURE 17

BPI Binds to LPS in the Presence of Serum



19/19

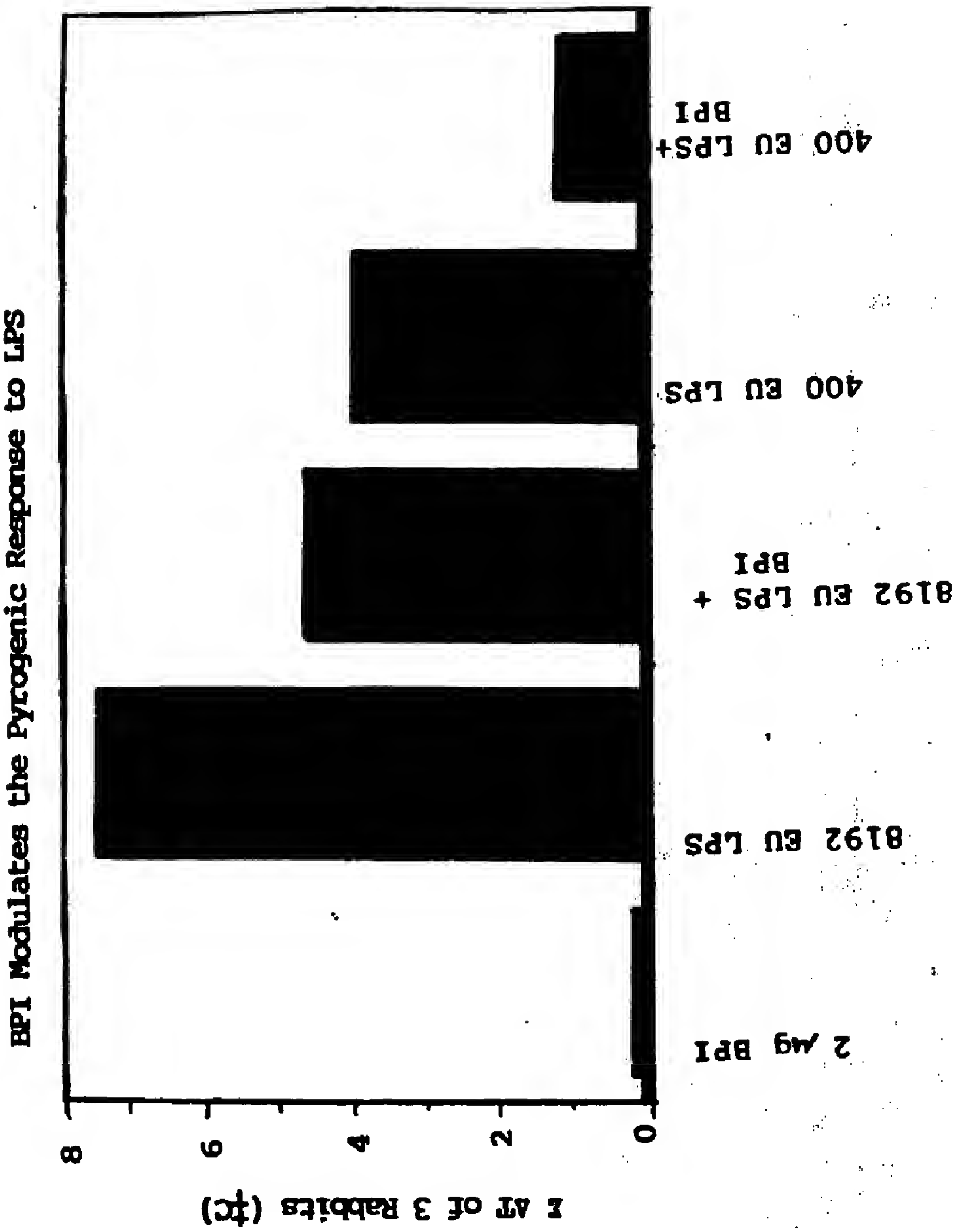


FIGURE 18

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US90/00837

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC
IPC (5): A61K 35/14, 37/02
U.S. CL: 424/101, 514/2,21, 530/829

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
U.S.	424/101; 514/2,21; 530/829

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

CAS, BIOSIS, APS

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y A	The Journal of Biological Chemistry, Volume 254, No. 21, issued 10 November 1979, P. Eisbach J. Weiss, R. Franson, S. Beckerdite-Quagliata, A. Schneider, and L. Harris, "Separation and Purification of a Potent bactericidal/permeability Increasing Protein and a Closely Associated Phospholipase A2 from Rabbit Polymorphonuclear Leukocytes", pages 11000-11009. See the abstract.	15-16, 29-36, 40-43 1-14, 17-28, 37-39
Y,P A,P	The Journal of Immunology, Volume 142, No. 8, issued 15 April 1989, B. Mannion, E. Kalatzis, J. Weiss and P. Elsbach, "Preferential Binding of the Neutrophil Cytoplasmic Granule-Derived Bactericidal/Permeability Increasing Protein to Target Bacteria: Implications and Use as a Means of Purification", pages 2807-2812, see pages 2811-2812.	40-43, 15-16, 29-36 1-14, 17-28, 37-39
Y A	The Journal of Biological Chemistry, Volume 262, No. 31, issued 05 November 1987, C. Ooi, J. Weiss, P. Elsbach, B. Frangione, and B. Mannion, "A 25-kDa NH2-Terminal Fragment Carries All the Antibacterial Activities of the Human Neutrophil 60-kDa Bactericidal/Permeability Increasing Protein", pages 14891-14894. See the abstract.	15-16, 30-36 1-14, 17-28, 37-39

* Special categories of cited documents:[10]

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed
- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search
27 APRIL 1990

Date of Mailing of this International Search Report
04 JUN 1990

International Searching Authority
ISA/US

Signature of Authorized Officer
Veronica White for
JEAN C. WITZ

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	The Journal of Biological Chemistry, Volume 263, No. 27, issued 25 September 1988, P. Tobias, J. Mathison and R. Ulevitch, "A Family of Lipopolysaccharide Binding Proteins Involved in response to Gram-negative Sepsis", pages 13479-13481. See the abstract.	1-14, 17-28, 37-39
A	The Journal of Clinical Investigation, Volume 69, issued April 1982, J. Weiss, M. Victor, O. Stendhal and P. Elsbach, "Killing of Gram-Negative Bacteria by Polymorphonuclear Leukocytes", pages 959-970. See the abstract.	1-14, 17-28, 37-39
A	The Journal of Clinical Investigation, Volume 76, issued July 1985, J. Weiss, L. Kao, M. Victor and P. Elsbach, "Oxygen-Independant Intracellular and Oxygen-Dependant Extracellular Killing of Escherichia coli S 15 by Human Polymorphonuclear Leukocytes", pages 206-212. See the abstract.	1-14, 17-28 37-39
A	Biological Abstracts, Volume 83, No. 9, issued 01 May 1987 (Philadelphia, Pennsylvania, USA), J. Weiss and I. Olsson, "Cellular and Subcellular Localization of the Bactericidal/Permeability Increasing Protein of Neutrophils", Ref. No. 86173, Blood, Volume 69, No. 2, issued 1987, pages 652-659. See the abstract.	1-14, 17-28 37-39